EFFECTS OF NATURAL PRODUCTS ON THE ACTIVITY OF HUMAN TOPOISOMERASE II

By

Kendra Raychell Vann

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Approved:

Neil Osheroff, Ph.D.

John D. York, Ph.D.

Charles R. Sanders, Ph.D.

Nicholas J. Reiter, Ph.D.

Katherine L. Friedman, Ph.D.

To my parents, Clifford and Frankie Vann, for your untiring prayers and support throughout my life.

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LIST OF ABBREVIATIONS

A	alanine
ATP	adenosine triphosphate
bp	base pair
C	cysteine
CA	caffeic acid: 3,4-dihydroxyphenylpropionic acid
CC	catalytic core: human topoisomerase IIα (residues 431-1193)
DMPE	3,4-dimethoxyphenylethanol
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin gallate
ET-1	N-methyl-5-demethyl ellipticine
ET-2	2-methyl-N-methyl-5-demethyl ellipticinium iodide
EtBr	ethidium bromide
Etop	etoposide
EVOO	extra virgin olive oil
FI	Form I: negatively supercoiled DNA
FII	Form II: nicked plasmid DNA
FII	Form III: linear plasmid DNA
[γ-^{32}P]ATP	adenosine triphosphate radiolabeled with 32P on gamma phosphate
GE-1	3,6-dihydroxy-2-propylbenzaldehyde
GE-2	2-hydroxymethyl-3-propylcyclohexa-2,5-diene-1,4-dione

HCl	hydrochloric acid
HMPE	4-hydroxy-3-methoxyphenylethanol
HT	hydroxytyrosol: 3,4-dihydroxyphenylethanol
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
kDNA	kinetoplast DNA
$K_3Fe(CN)_6$	potassium ferricyanide
Lk	linking number
Mt	mutant
MLL	mixed lineage leukemia gene
MWCO	molecular weight cut-off
NaOAc	sodium acetate
NaOH	sodium hydroxide
Ni-NTA	nickel-nitrilotriacetic acid
OE	oleuropein
OLE	olive leaf extract
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ProK	proteinase K
Rel	relaxed
RT	room temperature
SC	supercoiled

(-)SC	negatively supercoiled
SDS	sodium dodecyl sulfate
TIIα	human topoisomerase IIα
Top2αΔ1175	human topoisomerase IIα (residues 1-1175)
Tw	Twist
TY	tyrosol: 4-hydroxyphenylethanol
VERB	verbascoside
WT	wild-type
Wr	writhe

CHAPTER I

INTRODUCTION

DNA Topology

DNA Structure and Implications of DNA Topology

Deoxyribonucleic acid (DNA) is a molecule primarily located in the nucleus of a cell that carries genetic instructions used in the cellular development, function, and reproduction of all known living organisms and many viruses.

DNA was first identified in the late 1860s by Swiss chemist Friedrich Miescher.[1] Decades later, the research efforts of other scientists, such as Phoebus Levene and Erwin Chargaff, revealed additional details about the DNA molecule, including its primary chemical components and the ways in which the nucleotides paired with one another.[1,2] In 1953, James Watson and Frances Crick, along with Rosalind Franklin and Maurice Wilkins, were the first scientists to formulate an accurate description of the double-helical structure of DNA.[1-3] In a follow-up publication to Watson and Crick's famous paper describing the structure of DNA, they speculated on the implications of their discovery.[4] The various problems of disentangling DNA strands or duplexes in a cell would be all rooted in the double-helical structure of DNA. Watson and Crick realized that the cell would require a mechanism to overcome the inevitable topological issues

that would arise due to the inherent and newly discovered properties of their DNA structure.[4] Years later, James Wang discovered the first enzyme of many that had evolved to solve topological problems in the DNA. This enzyme was initially named the "ω protein" but was later called DNA topoisomerase I.[5] The details of how these enzymes work will follow after the inherent nature and challenges of DNA topology is described.

DNA Topology

It is important to first understand the nature of DNA structure that occurs in the cell. A typical DNA molecule consists of two complementary polynucleotide strands that are repeatedly intertwined every 10-10.5 bp into a double helix (Figure 1). In general, the length of chromosomal DNA in a cell far exceeds that of the cell it resides in. For example, the human genome contains approximately 2 meters of DNA, that must be compressed into the nucleus, which is only 5-10 μm in diameter.[6] Thus, a number of mechanisms are employed to extensively organize the DNA into higher order conformations to efficiently compact all the information needed to carry out cellular processes (Figure 2).

DNA is globally underwound by 6% in almost all living organisms.[7-13] This is important because duplex DNA is the storage form of the genetic information. In order to replicate or express this information, the two strands of DNA must be separated to gain access to this genetic material. The fact that DNA is globally slightly underwound facilitates strand separation due to the decreased amount of energy needed to break the hydrogen bonds between the bases of DNA.[9,10,14]

In all natural DNA, free rotation of the DNA ends is restricted, due to being typically circular in prokaryotes or tethered to the chromosomal scaffold in eukaryotes.

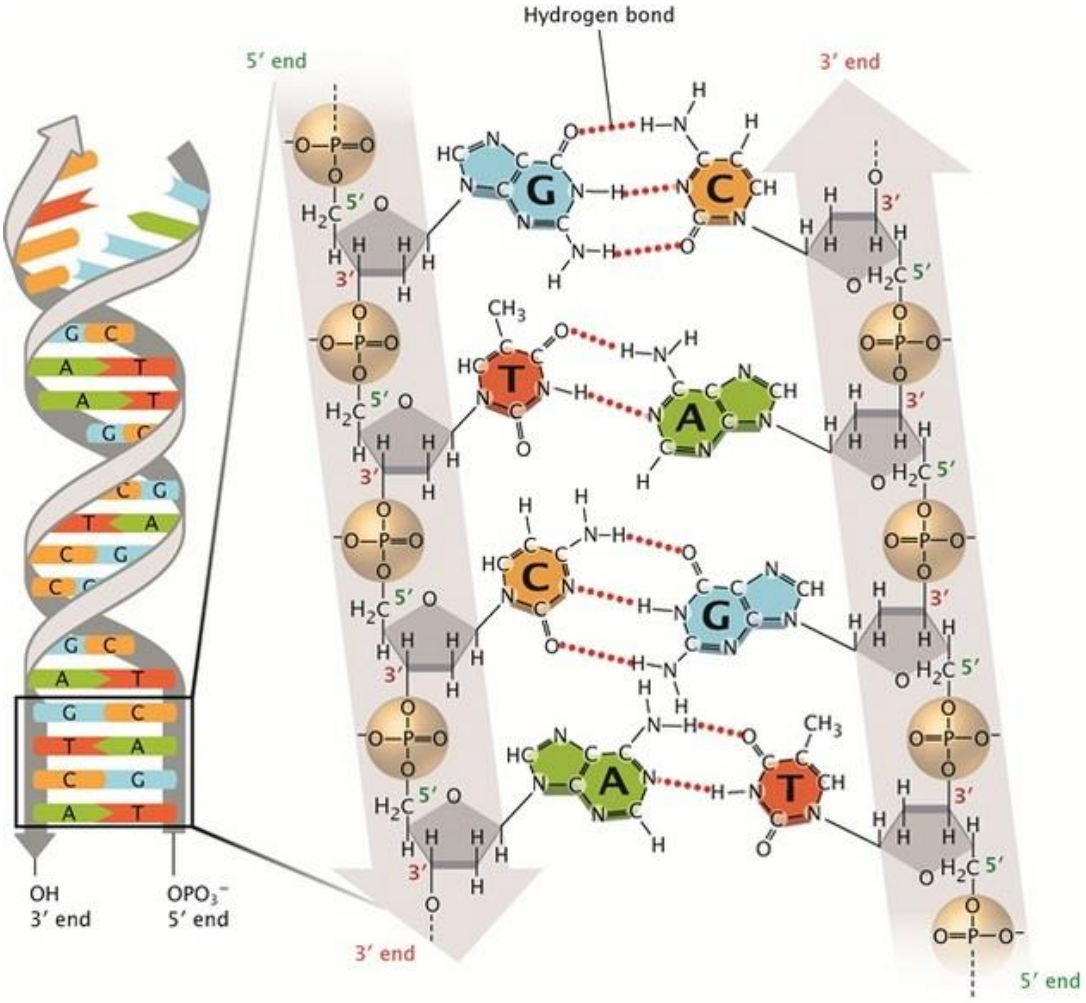


Figure 1. Structure of DNA. A typical DNA molecule (2 nm in width) and consists of two complementary polynucleotide strands repeatedly intertwined every 10-10.5 bp into a double helix is shown to the left. The sugar-phosphate backbones (grey) run anti-parallel to each other, so that the 3' and 5' ends of the two strands are aligned. Within these two complementary strands two hydrogen bonds connect thymine (T) to adenine (A) while three hydrogen bonds connect guanine (G) to cytosine (C). Figure reproduced from Ref. 2.

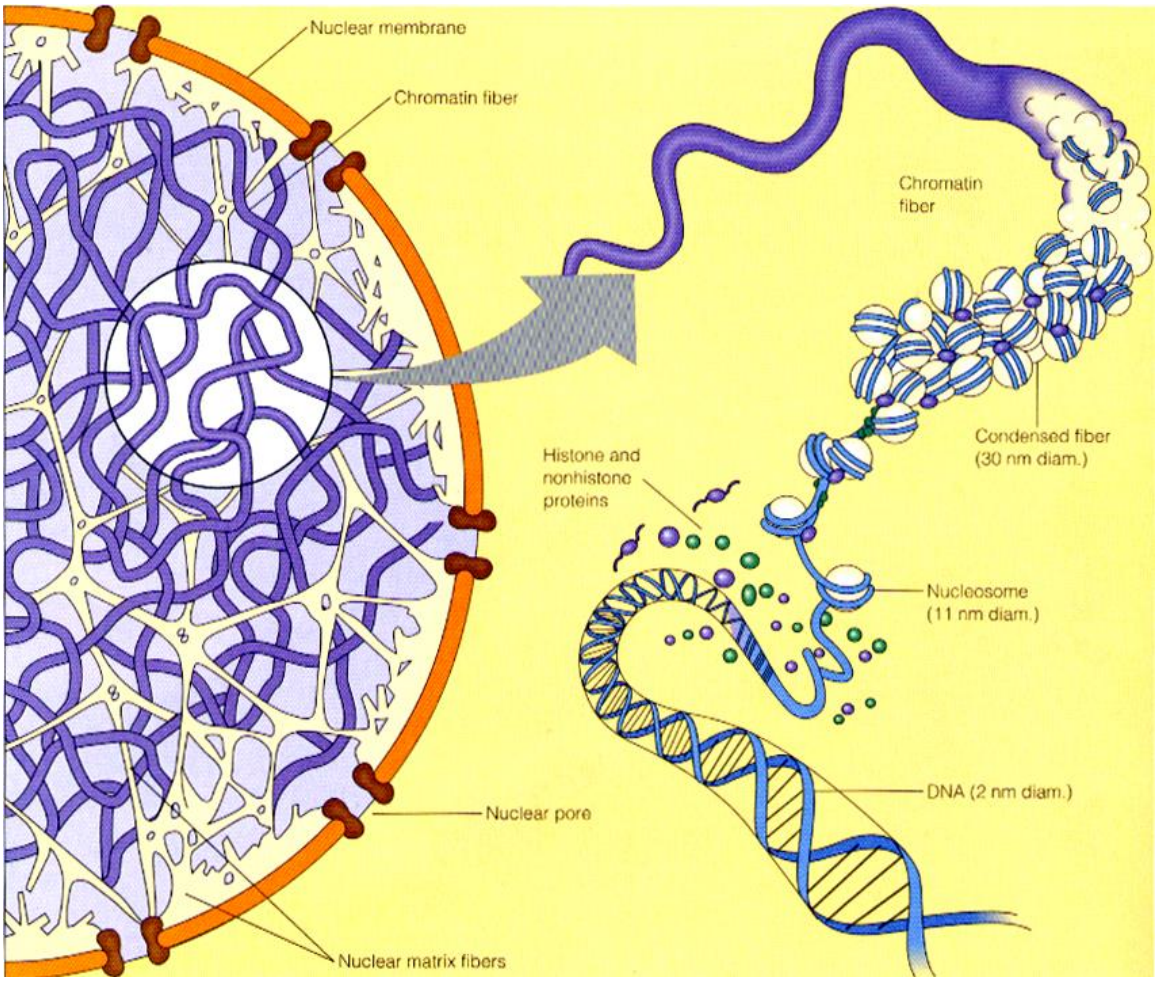


Figure 2. Model of DNA compaction inside of a cell nucleus. The cell must handle amounts of DNA that are many times longer than the cells they are in. Thus, DNA packaging must be very efficient while still allowing for DNA replication and transcription to occur. An example of the levels of compactions the DNA must undergo to fit into the nucleus is depicted. Figure reproduced from Ref.15.

These types of attachments hinder the free rotation of the DNA strands and are considered a closed system. Consequently, DNA underwinding or overwinding induce torsional stress within the DNA molecule during processes that occur to replicate the DNA in the cell (Figure 3). When the stress is freely distributed along the DNA, some of it is converted to axial stress within the DNA duplex. Superhelical twists are formed from the DNA writhing about itself through the redistribution of stress. Hence, DNA under torsional and axial stress is referred to as supercoiled DNA.[7,10,13,16] Accordingly, underwound and overwound DNA molecules are called negatively and positively supercoiled DNA, respectively.

While the genetic information encoded in DNA is a one-dimensional array of bases, the three-dimensional structure of the genetic material controls how this information is replicated, expressed, and recombined in the cell.[17] With the tight constraints of a closed system, the structure of DNA affects virtually every nucleic acid process that requires the double helix to be opened or moved within the cell.[13,17,18] Topological relationships of the DNA, such as overwinding, underwinding, knots, and tangles arise primarily from the fact that the two DNA strands are repeatedly intertwined. These topological relationships are defined as those that can be altered only by breaking one or both strands of the double helix.[13,17,18]

Parameters of Supercoiled, Knotted, and Tangled DNA

Supercoiled DNA has three fundamental parameters that are changed upon resolving the tension in the DNA duplexes: twist (*Tw*), writhe (*Wr*), and linking number (*Lk*). *Tw* represents the total number of double helical turns in a given segment of DNA.

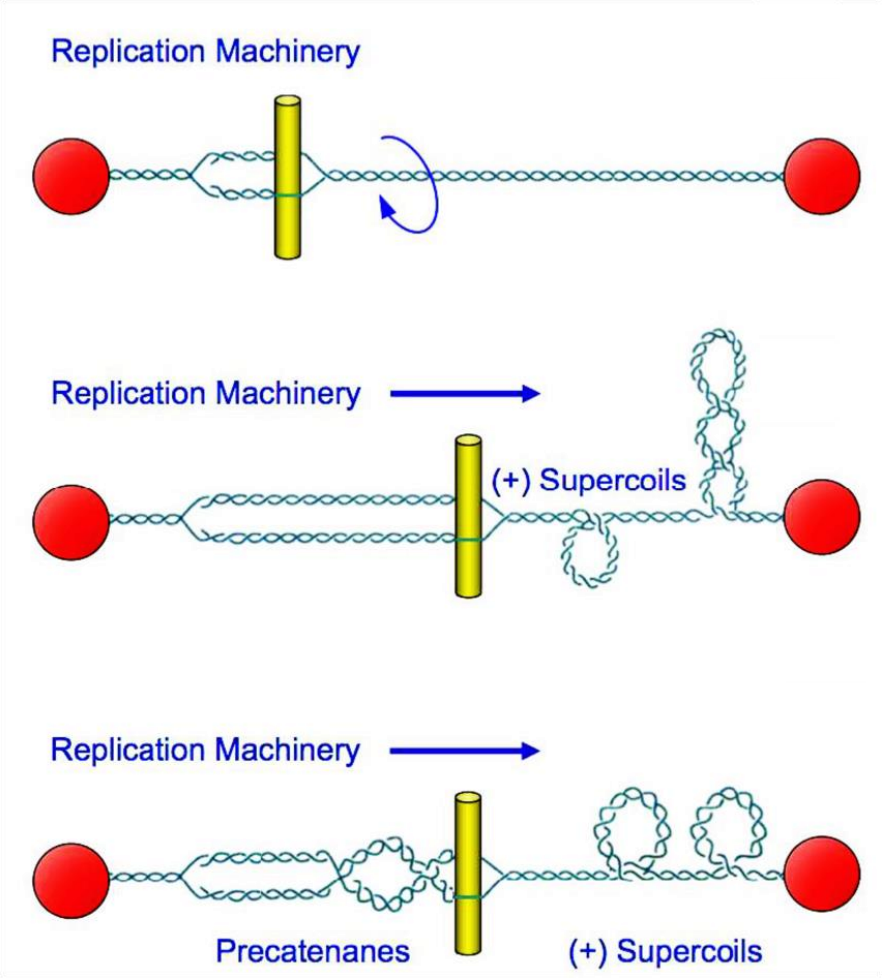


Figure 3. Topological challenges of DNA due to cellular processes. Nuclear processes induce changes in DNA topology. DNA replication is used as an example. Although chromosomal DNA is globally underwound in all cells, the movement of DNA tracking systems generates positive supercoils. (Top) The ends of chromosomal DNA are anchored to chromosome scaffold (represented by the red spheres) and are not free to rotate. Therefore, the linear movement of tracking systems (such as the replication machinery represented by the yellow bars) through the immobilized double helix compresses the turns into a shorter segment of the genetic material and induces acute overwinding (*i.e.* positive supercoiling) ahead of the fork (Middle). In addition, the compensatory underwinding (*i.e.* negative supercoiling) behind the replication machinery allows some of the torsional stress that accumulates in the pre-replicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes (Bottom). If these precatenanes are not resolved, they ultimately lead to the formation of intertwined (*i.e.* tangled) duplex daughter chromosomes. Figure reproduced from Ref. 19.

Wr represents the number of times the double helix crosses itself if the molecule is projected in two dimensions. *Lk* is a numerical term that describes the sum of the twist and the writhe and represents the total linking within a DNA molecule. These properties of supercoiled DNA can be mathematically explained as $Lk = Tw + Wr$. The only way to change *Lk* is to introduce a break in one or both DNA strands, rotate the two DNA strands relative to each other, and reseal the break.[6,13,17 18]

Knots and tangles are controlled by two fundamental parameters: *Wr* and *Lk.* DNA crossovers that are present in knots and tangles are controlled by *Wr* and cannot be freely interconverted from *Tw* to *Wr*, as in the case of supercoiled DNA. The *Lk* of knotted or tangled DNA can only be changed by breaks introduced in both strands of DNA.[13,17,18]

Challenges of DNA Topology

DNA supercoiling is important in a number of biological processes, such as compacting DNA and regulating access to the genetic information.[20,21] Separation of the two strands of the double helix must occur for transcription and replication to be carried out by RNA and DNA polymerases, respectively. As the two DNA strands are unwound by a polymerase-associated helicase, compensatory over- and under-winding occurs, due to the lack of free-end rotation. The replication or transcription machinery leads to positive supercoiling ahead of the fork and negative supercoiling behind (Figure 3).[13,18,22-24] Because the ends of DNA are fixed in space, critical cellular processes such as replication and transcription would stall before completion without a mechanism to relieve the torsional stress. Furthermore, torsional stress ahead of DNA tracking systems

is translated behind the fork and leads to the formation of precatenanes (Figure 3). Precatenanes link sister chromatids after replication and must be resolved in order for the chromosomes to be separated during mitosis.[9-11,13,18,23] Accordingly, just as negative supercoiling can facilitate normal DNA processes, positive supercoiling can inhibit and ultimately block them if not alleviated.[9-11,13,14,18]

Furthermore, during DNA replication, intermolecular links (catenanes) are formed between daughter DNA molecules. Unless these tangled DNA molecules can be decatenated, daughter chromosomes cannot be segregated during mitosis or meiosis. Likewise, intramolecular knots can be formed within the same DNA molecule and are generated during recombination. DNA knots block essential nucleic acid processes, because they make it impossible to separate the two strands of the double helix. Consequently, DNA knots and tangles can be lethal to cells if they are not resolved.[10,11,13,18,23]

DNA Topoisomerases

Enzymes Evolved to Resolve the DNA Topological Challenges

Topoisomerases are nature's solution to the topological complexities that occur in DNA. These enzymes regulate the topological problems that naturally occur in DNA during cellular processes. Accordingly, DNA topoisomerases are essential for the survival of all organisms and alter DNA topology by generating transient breaks in the double helix.[19,23,25-28] By creating transient breaks in the DNA, topoisomerases can then regulate the topology of the DNA through three main tasks: 1) maintaining appropriate chromosome topology, 2) removing positive supercoils, and 3) unlinking sister

chromatids during replication, strand separation, and cell division (Figure 4).[19,23,25-28] Thus, topoisomerases play essential roles in proliferating cells.

All topoisomerases transiently cleave the DNA backbone by a nucleophilic attack using a catalytic tyrosine residue. The tyrosine residue then becomes covalently linked to the phosphate end of the DNA break. This covalent enzyme-DNA complex is known as the "cleavage complex" and is a hallmark catalytic event of topoisomerases. Religation of the DNA is highly favored and leaves the DNA sequence unchanged following the catalytic cycle of the enzyme.[19,23,25-29]

Topoisomerases can be separated into two major classes, type I and type II. These classes are distinguished by the number of DNA strands that are cleaved and ligated during the catalytic cycle of their respective enzymes.[19,23,25-28,30-35] The specific class of the enzyme is often denoted by numbers at the end of the name, with odd numbers signifying Type I enzymes and even numbers indicating Type II enzymes (Figures 4 and 5). Type I topoisomerases transiently cleave a single strand of the DNA duplex, while type II topoisomerases cleave both strands. Furthermore, topoisomerases are further divided into subtypes (A, B, or C) comparing their sequence similarity, DNA interaction and/or global structure.[19,23,26-28,31-34,36]

Type I Topoisomerases

There are two main subclasses of type I topoisomerases: type IA and type IB (Figure 5). Type I topoisomerases act as monomers and alter the topology of supercoils by creating transient single-stranded breaks in the DNA, followed by passage of the opposite intact strand through the break (type IA) or by controlled rotation (swivel) of the

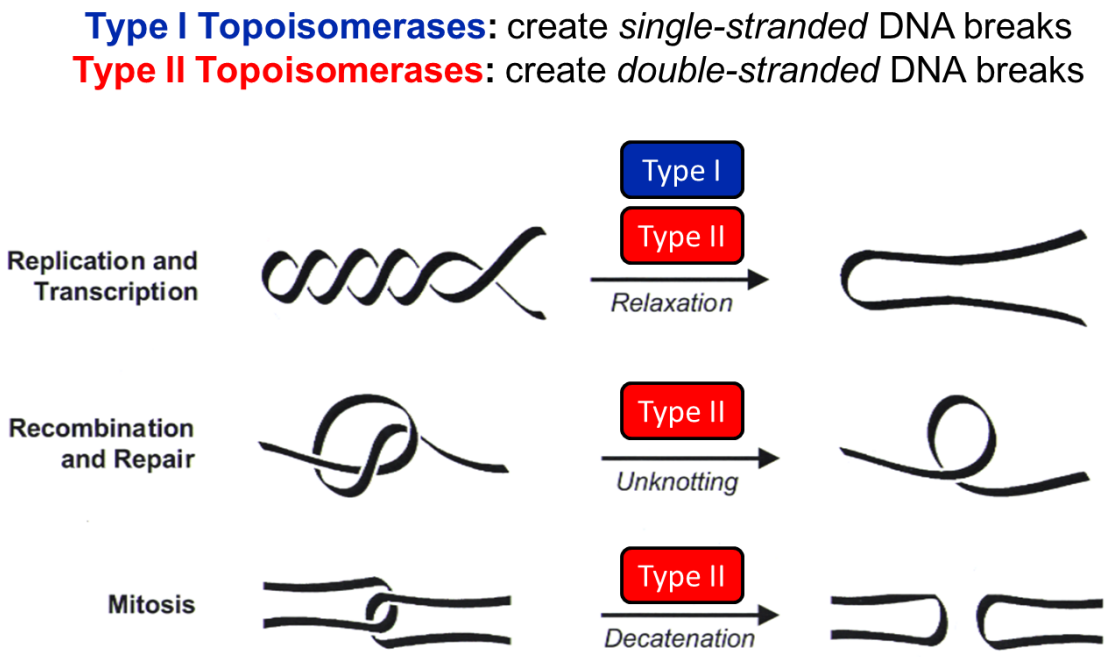


Figure 4. Topological challenges overcome by topoisomerases. A schematic is shown to indicate some of the cellular process in which topological challenges of DNA can occur and the role of topoisomerases to resolve the problem. (Top) During replication and transcription, the movement of the growing fork induces the formation of supercoils ahead of the fork and precatenanes behind the fork. In order for extensive DNA synthesis to proceed, the positive supercoils must be removed (relaxed). During recombination and repair, knots can form in the DNA and have to be unknotted (Middle). During mitosis, sister chromatids are tangled together. If the daughter chromosomes are not decatenated, cells will die of mitotic failure. To resolve these topological problems associated with DNA, cells possess enzymes called topoisomerases that function by creating transient breaks in the DNA to relax, unknot or decatenate the DNA. Figure produced by the Osheroff lab.

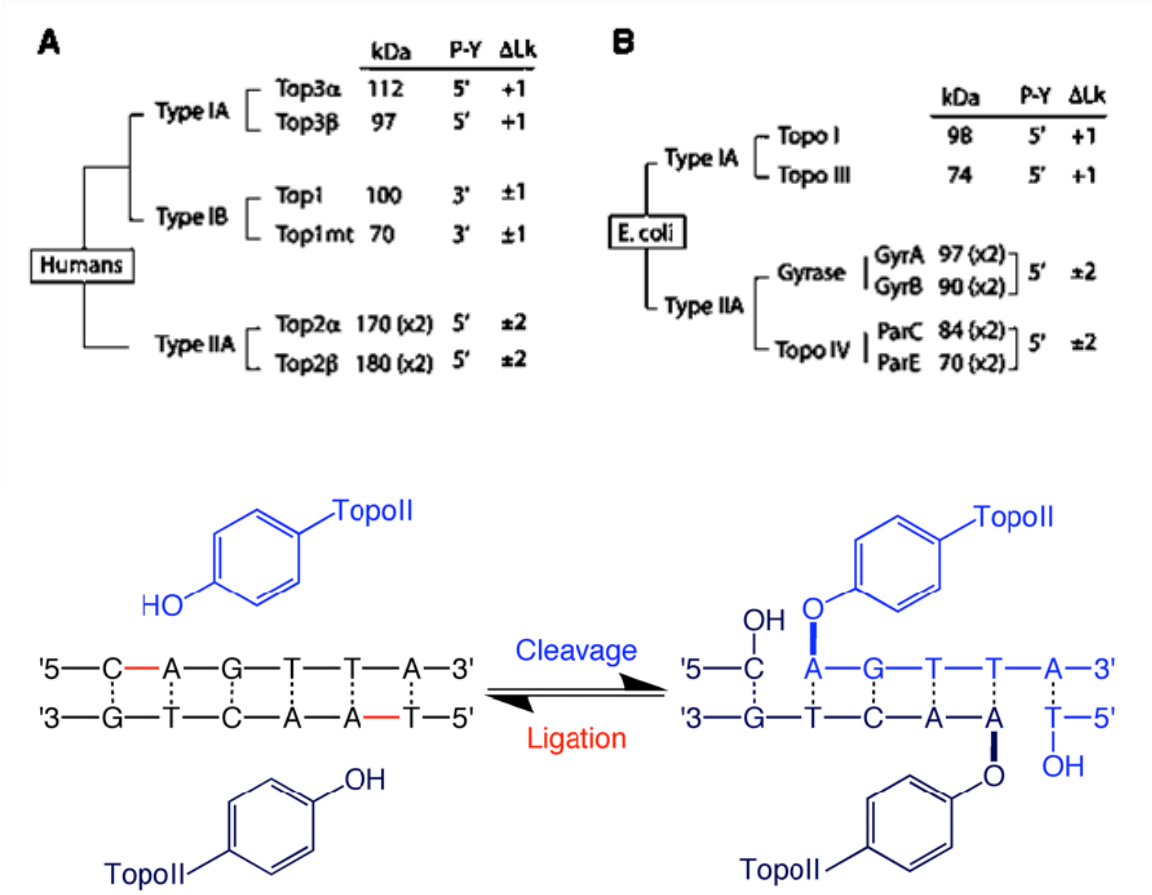


Figure 5. Generation of topoisomerase-DNA cleavage complex. (Top) Classification of human (A) and *E. coli* (B) DNA topoisomerases. The molecular masses (kDa), the prime end that the catalytic residue of the enzyme covalently links to (P-Y), and the change in linking number induced by the enzyme (ΔLk) are indicated. (Bottom) Double-stranded DNA cleavage intermediates of the topoisomerase II are shown. The scissile bonds (red) are located four bases apart on opposite strands of the double helix. During cleavage, the active site tyrosine residue of each topoisomerase II protomer subunit becomes covalently linked to the newly generated 5'-terminal phosphate moiety on each strand. Ligation represents the reverse of this process and leaves the DNA product chemically unchanged from the initial substrate. Top figure adapted from Ref. 26 and bottom figure reproduced from Ref. 19.

helix around the break (type IB). Type IA topoisomerases require divalent metal ions for DNA scission and attach covalently to the 5'-terminal phosphate of the DNA. In contrast, type IB enzymes do not require divalent metal ions and covalently link to the 3'-terminal phosphate.[18,19,23,26,27,29,31,32,36] Type IA and IB topoisomerases differ in both structure and function.[26,27,36] While Type IB enzymes can relax both positive and negative supercoils, Type IA can only relax negative supercoils.[19,23,26,27,31,32,34,36]

Due to their single-stranded cleavage reaction mechanism, type I topoisomerases usually remove one supercoil at a time and change the *Tw* of the supercoil, altering the *Lk* by one. Therefore, Type I enzymes can modulate DNA under- and overwinding, but cannot remove knots or tangles from duplex DNA (Figure 4).[13,18] These topological forms of DNA require type II topoisomerases, which I will focus on in the research presented.

Type II Topoisomerases

Type II eukaryotic enzymes act as dimers and relax (or in the case of gyrase induce) supercoils, untangle (decatenate), and unknot DNA due to their ability to cleave both strands of the double helix (Figures 4 and 5). These enzymes require ATP and divalent metal ions for overall catalytic activity.[37-39] Briefly, type II topoisomerases modulate DNA topology by generating a transient double-stranded break in the DNA backbone, passing a separate double helix through the opening, and resealing the break (Figure 6). During the intermediate step of the catalytic cycle, type II enzymes covalently attach to the accessible 5'-terminal phosphates (4 base pairs apart on alternating strands)

created by the nucleophilic attack by the catalytic tyrosine residue of each monomer forming the "covalent complex" (Figures 5 and 6).[19,23,25-27,31]

As a result of their double-stranded DNA passage mechanism, type II topoisomerases act solely on DNA writhes and remove two supercoils at a time. Therefore, they alter the *Lk* in steps of two. Because type II enzymes act on DNA writhes, they can remove knots and tangles from the genetic material.[13,18] Importantly, at the end of replication, type II enzymes enable the segregation of newly replicated chromosomes.[19,28,40,41]

All living organisms encode at least one type II topoisomerase.[26,31,42-45] Prokaryotic enzymes function as A_2B_2 heterotetramers and eukaryotic enzymes as homodimers. The prokaryotic and eukaryotic type II enzymes are homologous; in the eukaryotic type II homodimers (A_2), the A and B subunits of the bacterial type II enzyme merge into a single subunit.[8,13,23,31,34,46-48]

Lower eukaryotes only encode one type II topoisomerase, while mammals express two isoforms of topoisomerase II, α and β. Human topoisomerase IIα and β are encoded by two separate genes (17q21–22 and 3p24, respectively) and differ in molecular mass (170 and 180 kDa, respectively). Both isoforms share extensive sequence identity (70%) and display similar enzymatic properties, but differ significantly in their expression, cellular regulation and functions.[8,26,44,49-53]

Expression of topoisomerase IIα is linked to cellular growth and is essential for the survival of proliferating cells.[18,19,25,27,35,48,54] Overexpression of the enzyme is also monitored as a cancer cell marker.[27,28,36,55-57] Protein concentrations of topoisomerase IIα are regulated over the cell cycle, increasing 2- to 3-fold during the G2/M phases.

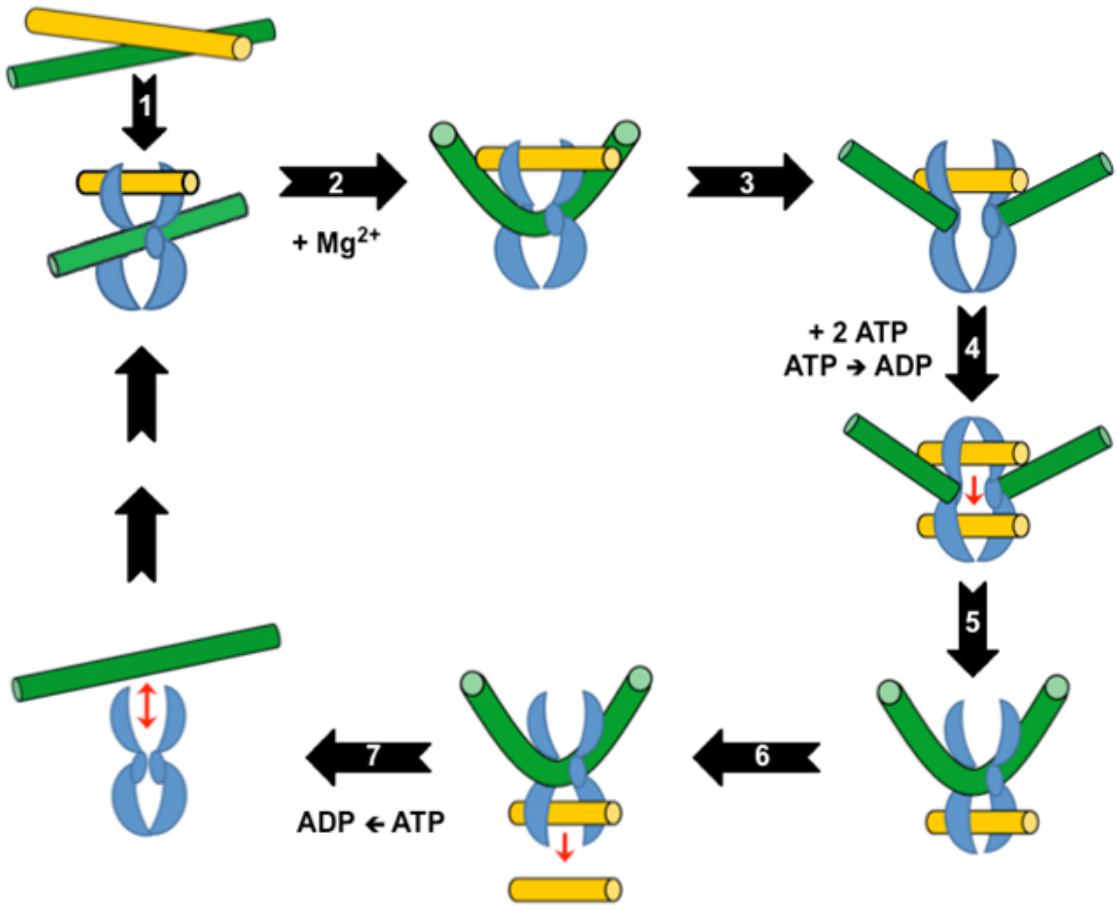


Figure 6. Catalytic cycle of type II topoisomerases. In order for topoisomerase II to relax, untangle, or decatenate DNA: 1) the enzyme binds to a DNA crossover, positioning the G-segment (green) of DNA in the DNA gate and the T-segment of DNA (yellow) within the N-gate. 2-3) In the presence of a divalent cation, the enzyme bends the G-segment, then cleaves and covalently attaches to DNA through the active site tyrosine residue. This covalent enzyme-DNA intermediate is known as the cleavage complex. 4) Through binding of ATP the N-gate closes and hydrolysis of 2 ATP induce conformational changes. These changes allow the enzyme to pass an intact T-segment through the cleaved G-segment. 5) The enzyme then religates the G-segment and 6) releases the T-segment 7) Hydrolysis of another ATP completes the catalytic cycle and allows the enzyme to releases the G-segment. Figure reproduced from Ref. 58.

Levels of the α isoform are virtually non-existent in quiescent and differentiated tissues, while rapidly proliferating cells contain ~500,000 molecules.[28,52] The α isoform is associated with replication forks and remains tightly bound to chromosomes during mitosis. It also plays critical roles in DNA replication and recombination, and is required for proper chromosome organization and segregation.[23,28,30,47]

Expression of topoisomerase IIβ cannot compensate for the loss of topoisomerase IIα. Although the β isoform is not required at the cellular level, it is essential for neuronal development.[13,27,31,53,59,60] High levels of the β isoform are found in most cell types, independent of proliferation status.[53,54,61] Topoisomerase IIβ dissociates from chromosomes during mitosis but appears to play an important role in the transcription of hormonally and developmentally regulated genes.[53,62-64] Despite the differences between the α and β isoforms of topoisomerase II, these enzymes are mechanistically similar and will be collectively referred to as topoisomerase II or type II enzyme, unless otherwise noted.

Structural Domain Organization of Eukaryotic Topoisomerase II

On the basis of detailed structural and biochemical information, topoisomerase II enzymes are architecturally similar. Eukaryotic type II enzymes are homodimers with each protomer (~170 kDa) arranged symmetrically (Figure 7). Each protomer contains three main regions: N-terminal region (N-gate), cleavage/ligation region (DNA-gate or catalytic core), and C-terminal region (C-gate). The N-gate includes the Gyrase, Hsp90, Histidine Kinase, MutL (GHKL) domain and the transducer domain, which bind ATP and transduce hydrolysis signals to downstream regions of the enzyme, respectively.

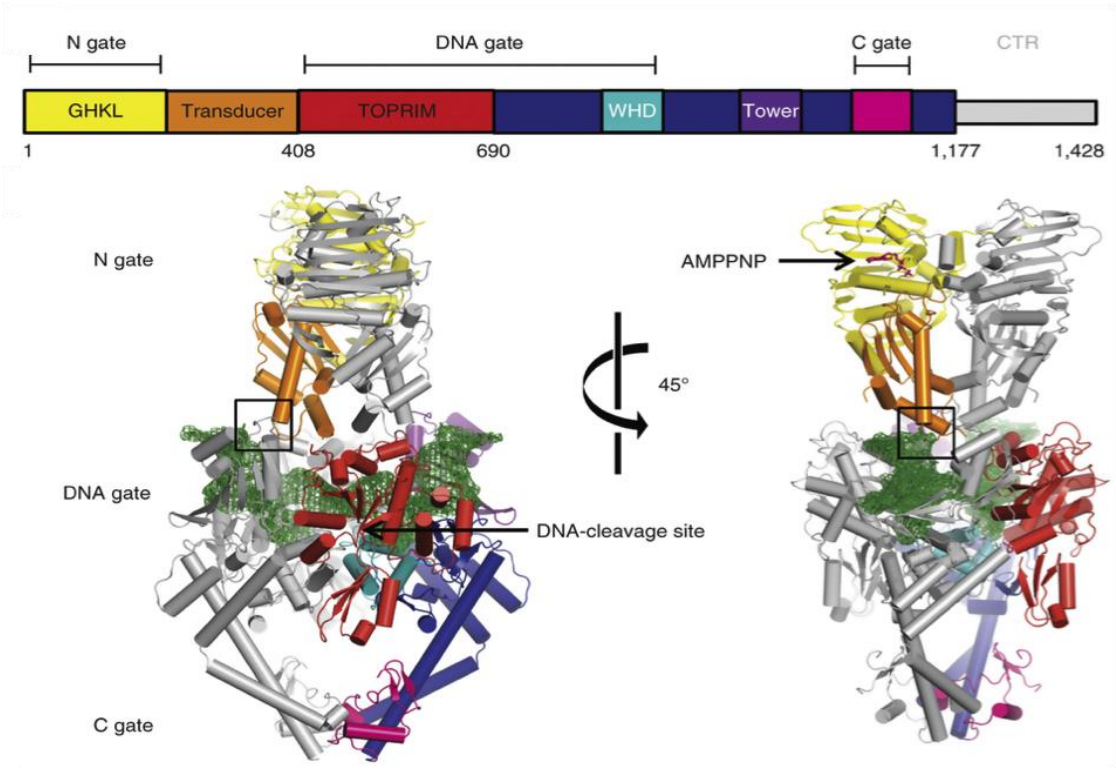


Figure 7. Domain organization and structure of eukaryotic topoisomerase II. A diagram depicting the functional regions of topoisomerase II are colored and labeled above. A model of the ternary complex of yeast topoisomerase IIα is depicted below. One topoisomerase II protomer is shaded gray and the other is colored as depicted by the diagram. The green indicates the cleaved DNA contoured in $2F_o - F_c$ density. The C-terminal region (CTR) is not present in the structure. Figure reproduced from Ref. 65.

The DNA gate contains the topoisomerase/primase (TOPRIM) domain that coordinates the divalent metal ions, and the winged-helix domain (WHD) that includes the active site tyrosine. The C-terminal domain (CTD) contains nuclear localization signals and post-transcriptional modification sites. The CTD is largely disordered and less conserved among the type II enzymes.[66-68] A schematic and structure of the domain organization can be seen in Figure 7.

Catalytic Mechanism of Topoisomerase II

The multiple subunits of type II topoisomerases act through a complex mechanism to physically move one DNA duplex through another in order to help resolve DNA topological problems (Figures 6 and 7).[66,68-71] In the DNA cleavage reaction, the dimer binds two segments of DNA at a crossover. The first segment bound by the enzyme is the double helix that will be cleaved by the enzyme and is referred to as the "Gate-" or "G-segment," due to the location within the DNA-gate.[72] The second segment is the double helix positioned in the N-gate that will be transported through the transiently cleaved G-segment and is referred to as the "Transport-"or "T-segment."[70] Divalent metal ion(s) are then coordinated within the TOPRIM domain (located in the DNA-gate), and the type II topoisomerases sample the DNA for malleability.[38,65,72,73] Sequences within the G-segment that can be distorted to an ~150º angle are bent by the enzyme.[45,72] Cleavage of the G-segment is then catalyzed by a nucleophilic attack on the DNA backbone, at sites located four bases apart on opposite strands of the double helix, using the active site tyrosine residue of each topoisomerase II protomer.[74-76] Subsequently, the topoisomerase II protomers become covalently linked to the newly generated 5′-

terminal phosphate moiety on each strand of the G-segment through a trans-esterification reaction (Figures 5 and 6). This assembly is called the "cleavage complex." The topoisomerase II protomers bind two ATP within the ATPase domain (located in the N-gate) and close the N-gate.[69,74] Upon ATP hydrolysis, signals are transduced to downstream regions of the enzyme so that the T-segment is then transported through the DNA gate.[69,71,77] The G-segment is then resealed, and the T-segment is released.[46,69,71,74,76,78] After another ATP is hydrolyzed and the G-segment is released, enzyme is free to capture another crossover.[69,74,79]

Cleavage-Ligation Balance

The covalent enzyme-DNA linkage formed during DNA cleavage plays two important roles in the topoisomerase II reaction mechanism.[8,34,74,80,81] First, it conserves the bond energy of the DNA backbone. Second, it maintains the integrity of the genetic material during the cleavage event, because it does not allow the cleaved DNA chain to dissociate from the enzyme.[34,80,82,83] The cleavage complex is central to the catalytic cycle of the enzyme. The DNA cleavage/ligation equilibrium of the enzyme greatly favors ligation.[8,26,27,34,74,84-86] Topoisomerase II-DNA cleavage complexes normally are short-lived and are readily reversible.[19,47,48,87] Alterations by compounds or DNA lesions that increase the longevity of cleavage complexes have serious cellular consequences.[19,25-27,45]

Many type II topoisomerases are essential in proliferating cells. However, since these enzymes generate double-stranded DNA breaks as part of their reaction mechanism, they are intrinsically dangerous proteins. Thus, while essential to cell viability, the

enzymes also have the ability to fragment the genome (Figure 8). Because of this dual persona, levels of cleavage complexes are normally maintained in a critical balance.[19,25,27,36,68]

When levels of cleavage complexes drop below threshold concentrations, daughter chromosomes remain entangled following replication. As a result, sister chromatids cannot properly segregate during mitosis and cells die due to catastrophic mitotic failure.[23,30,31,47,88,89] When levels of cleavage complexes rise too high, cells also die, but for different reasons. In this instance, cell death is due to the conversion of transient DNA cleavage intermediates to permanent strand breaks.[90,91] Permanent strand breaks are believed to be generated when replication forks, transcription complexes, or DNA tracking enzymes such as helicases attempt to traverse the covalently bound protein 'roadblock' in the genetic material.[19 ,56,91-95] The resulting damage and induction of recombination/repair pathways also can trigger mutations, chromosomal translocations, and other aberrations. When these permanent DNA breaks are present in sufficient numbers, they can overwhelm the cell and initiate cell death pathways.[19 ,56,90-95]

Topoisomerase II Inhibitors vs. Poisons

Compounds that alter topoisomerase II activity can be separated into two categories: topoisomerase II catalytic inhibitors and topoisomerase II poisons (Figure 8). Topoisomerase II catalytic inhibitors are chemicals that act by robbing the cell of the essential catalytic functions of the type II enzymes, and generally do not increase the concentration of cleavage complexes.[47,56,67,96,97] Inhibitors have been shown to act at a variety of steps of the topoisomerase II catalytic cycle, including DNA cleavage.

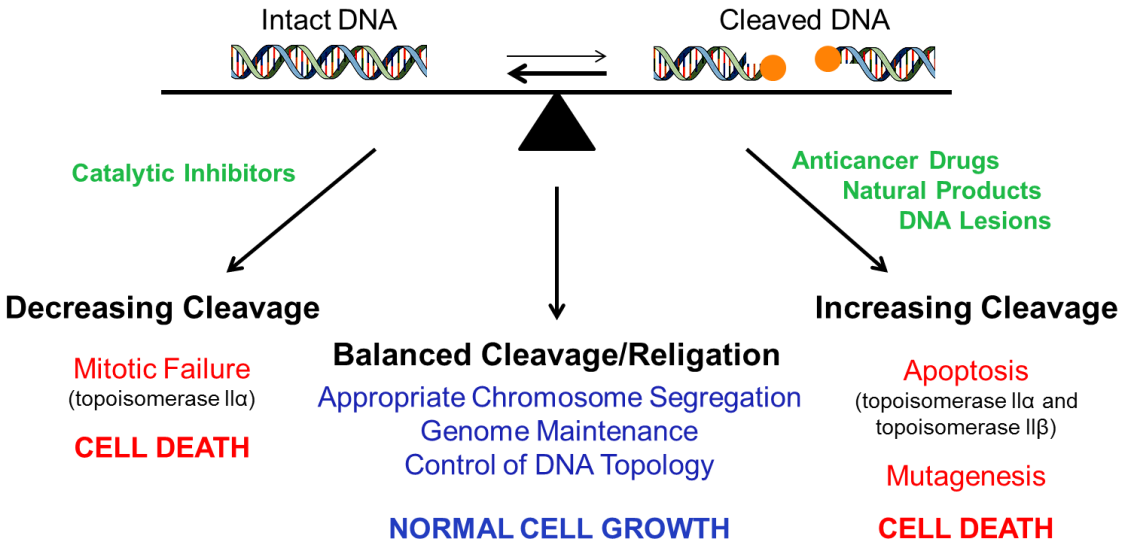


Figure 8. Shifting the equilibrium of topoisomerase II DNA cleavage. A balanced level of topoisomerase II-DNA cleavage complexes is required for the enzyme to perform its critical cellular functions (blue). If the level of topoisomerase II-DNA cleavage complexes falls too low (left arrow, red), cells are not able to untangle daughter chromosomes and ultimately die of mitotic failure. If the level of cleavage complexes becomes too high (right arrow, red), the actions of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks. The resulting DNA breaks, as well as the inhibition of essential DNA processes, initiate recombination/repair pathways and generate chromosome translocations and other DNA aberrations. If the strand breaks overwhelm the cell, they can trigger apoptosis. This is the basis of several widely prescribed anticancer drugs and natural products that target topoisomerase II. If the concentration of topoisomerase-mediated DNA strand breaks is too low to overwhelm the cell, mutations or chromosomal aberrations may be present in surviving populations. In some cases, exposure to topoisomerase II poisons has been associated with the formation of specific types of leukemia. Figure adapted from Ref. 98.

Compounds that interact at these steps can be categorized by their ability to 1) prevent binding of the enzyme to the DNA, 2) compete for ATP binding, 3) inhibit ATP hydrolysis, and 4) block DNA cleavage.[47,67,96,97]

Alternatively, chemicals that increase levels of topoisomerase II-DNA cleavage complexes are said to "poison" the enzyme and convert it into a cellular toxin that initiates the mutagenic and lethal consequences.[91,94] Thus, they are called "topoisomerase II poisons." [19,47,89] Topoisomerase II poisons interfere with the ability of the enzyme to religate cleaved DNA molecules.[19,47,81,91,99] Although some topoisomerase II poisons inhibit overall activity, the increased levels of cleavage complexes induced by these compounds in the cell is a "gain of function," which is a dominant phenotype.[48,87] All clinically relevant topoisomerase II-targeted drugs examined to date act as topoisomerase II poisons, and thus display anticancer properties as described in the topoisomerase II-targeted drugs section.[19,47,81,91,99]

Mechanisms of Topoisomerase II Poisons

Topoisomerase II poisons act by two distinct mechanisms. Compounds that operate by the first mechanism are referred to as "interfacial poisons." Interfacial poisons are chemicals that form non-covalent interactions with topoisomerase II at the protein-DNA interface within the vicinity of the active site tyrosine. They also interact with DNA within the enzyme-DNA ternary complex and inhibit ligation by intercalating into the double helix at the cleaved scissile bond.[26,47,48,56,81,87] A hallmark characteristic of interfacial poisons is their ability to present a physical barrier to ligation and act as "molecular doorstops."[56,81,89,100,101]

In contrast to interfacial topoisomerase II poisons, compounds that act by the second mechanism contain protein-reactive groups and are referred to as "covalent poisons." [19,48,57,67,87] Most covalent poisons incorporate sulfhydryl-reactive groups.[57,102-105] Covalent poisons act by adducting to the enzyme through an acylation reaction at amino acid residues outside of the active site.[57,104-107] Additionally, their ability to poison topoisomerase II can be abolished by reducing thiol nucleophiles of the compound, rendering them unable to adduct to the reactive residues in the enzyme.[84,103,105,106,108] Finally, compounds that act as covalent poisons enhance DNA cleavage when added to the protein-DNA complex, but display the distinguishing feature of inhibiting topoisomerase II activity when incubated with the enzyme prior to the addition of DNA.[58,84,103,104,109-113] Presumably, this may reflect the fact that closing the N-terminal protein gate prevents DNA binding.[104,111]

Previous studies have shown that covalent poisons can accommodate a greater range of structural alterations than interfacial poisons.[58,84,102-106,111-115] These findings suggest that covalent poisons act more as chemical modification reagents than "ligands" that require specific binding pockets on topoisomerase II. Furthermore, some covalent topoisomerase II poisons have been found to alter enzyme function, at least in part, by enhancing protomer crosslinking and N-terminal protein clamp closure.[102,104,107] These studies suggest a mechanistic basis for stabilizing pre-existing cleavage complexes, while preventing DNA binding of unoccupied enzymes.[19,57,67] However, additional possibilities cannot be excluded, and the detailed mechanisms by which covalent topoisomerase II poisons increase levels of DNA cleavage complexes have yet to be determined.

Notably, the actions of interfacial topoisomerase II poisons are not affected by reducing agents, such as dithiothreitol (DTT), and these compounds induce similar levels of enzyme-mediated DNA cleavage whether they are added to the enzyme-DNA complex or are incubated with the enzyme prior to the addition of DNA.[48,103,116,117]

Topoisomerase II-Targeted Drugs

Targeting Topoisomerase II

The immense interest in topoisomerase II in recent years derives not only from the recognition of their crucial role in managing DNA topology, but also from major advances in identifying targets of cytotoxic drugs. Notably, topoisomerase IIα is overexpressed in rapidly proliferating cells such as tumor and bacterial cells and is an ideal target for anticancer and antibacterial drugs. A wide variety of topoisomerase-targeted drugs, some of which are currently in widespread clinical use, have been discovered to generate enhancement of cytotoxic lesions by trapping the enzymes in covalent enzyme-DNA complexes, thus leading to cytotoxicity in rapidly proliferating cells. These topoisomerase II-targeted drugs function as topoisomerase II poisons and can be utilized as anticancer or antimicrobials chemotherapeutics.[19,26,36,47,56,76]

Clinically Relevant Topoisomerase II Poisons

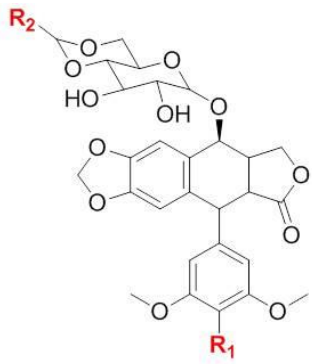
Topoisomerase II poisons represent some of the most successful and widely prescribed anticancer drugs worldwide.[19,28,36,48,56,57] Currently, six topoisomerase II-targeted agents are approved for use in the United States.[19,28,36,48,56,57,67,118] These drugs

encompass a group of naturally derived and synthetic compounds and are used to treat a variety of human malignancies (Figure 9). Notably, etoposide, doxorubicin, and their derivatives are highly active anticancer agents and are frontline therapies for a number of systemic cancers and solid tumors, including leukemias, lymphomas, sarcomas, breast cancers, lung cancers, neuroblastoma, and germ-cell malignancies.[19,28,36,56,57] Furthermore, mitoxantrone is used to treat breast cancer, acute myeloid leukemia (AML), and non-Hodgkin lymphoma, and amsacrine is used to treat relapsed acute myeloid leukemia.[28] Ultimately, half of all anticancer regimens include topoisomerase II-targeted drugs.[19,28,36,56,57]

Etoposide is by far the best-characterized topoisomerase II poison.[94,99,101,113,116,119-123] Research involving this drug has provided a knowledge-base that has paved the way for later work on other anticancer agents. Etoposide was one of the first chemotherapeutic drugs demonstrated to kill cells by targeting topoisomerase II and inhibiting the DNA ligation activity of the type II enzyme.[90,94,99,101,113,115,116,119-126] Etoposide is an interfacial topoisomerase II poison that enters the binary enzyme-DNA complex primarily through interactions with the protein.[116] Recent structural data with the drug has helped to reveal structure-function relationships with the topoisomerase II-DNA complex.[67,68,101,127-129]

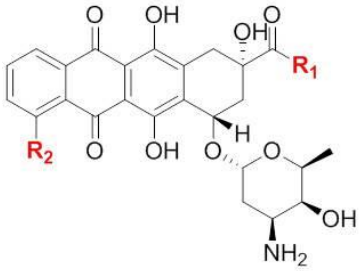
Non-Intercalating vs. Intercalating Topoisomerase II Poisons

Topoisomerase II poisons can be further subdivided into non-intercalating and intercalating poisons, with clinically relevant drugs in both categories. Non-intercalating topoisomerase II poisons include the epipodophyllotoxins, etoposide and teniposide, and



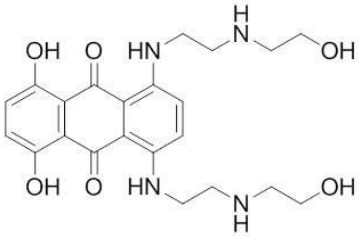
demethyl-epipodophyllotoxins

	R_1	R_2
Etoposide	OH	CH_3
Etoposide Phosphate	OPO_3H_2	CH_3
Teniposide	OH	

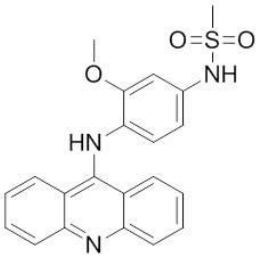


anthracyclines

	R_1	R_2
Doxorubicin	CH_2OH	OCH_3
Daunorubicin.	CH_2	OCH_3
Idarubicin	CH_2OH	H



Mitoxantrone



Amsacrine

Figure 9. Clinically relevant topoisomerase II anticancer drugs. The demethyl-epipodophyllotoxins etoposide, etoposide phosphate, and teniposide, the anthracyclines doxorubicin, daunorubicin, and idarubicin, and the anthracenedione mitoxantrone are approved for clinical use in the United States.

fluoroquinolones and have the ability to trap topoisomerase II-DNA covalent complexes. These non-intercalative compounds do not strongly interact with the DNA substrate, but mainly act through association with the protein.[101,130-132] Recent structural details reveal the detailed interactions between topoisomerase II, DNA, and the non-intercalative drug, etoposide. In the ternary complex, two etoposide molecules bind between the base pairs immediately flanking the two cleaved scissile bonds. This interaction is mediated by direct contacts with surrounding residues of topoisomerase II. Consequently, the protein-DNA-drug association stabilizes the cleavage complex by physically blocking the topoisomerase II-mediated resealing of DNA.[101]

The intercalators are chemically diverse and include compounds such as doxorubicin, daunorubicin, mitoxantrone, mAMSA, and a variety of other compounds that are not currently in clinical use (*ie.* amonafide and ellipticine).[56] Topoisomerase poisons that act as intercalators, usually contain a planar tri- or tetracycline ring base. The planar structure is usually positioned between the +1/-1 base positions within the cleaved G-segment, inhibiting the ability of the G-segment to religate. Anthracyclines (doxorubicin, daunorubicin, and their derivatives) can affect a broad range of DNA processes by intercalation and undergo redox reactions that generate reactive oxygen species (ROS), which have been implicated in their dose-limiting cardiotoxicity.[26,56]

Natural Products as Anticancer Drugs

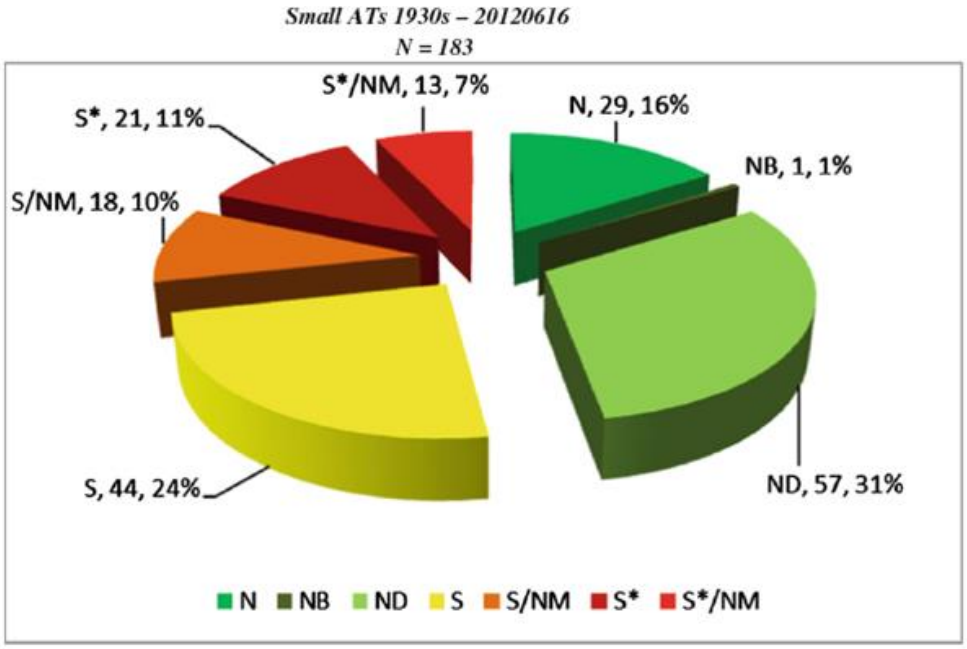
A "natural product" is generally regarded as being a secondary metabolite from a natural source. Natural products are organic substances (< 3 kDa) that are structurally diverse throughout nature. These compounds are derived from plants, microbes, and

marine sources, and can be either naturally active within the source to support the species survival or be a byproduct of the specie's metabolism.[133,134]

Humans have relied on natural products as a medicinal resource for thousands of years. Notably, plant-based drugs have formed the basis of traditional medicine that has been used for centuries in Egypt, China, and India.[134-136] In the majority of developing countries, particularly those in Asia, Africa, Latin America, and the Middle East, between 70-95% of citizens still use traditional and herbal medicines for primary health care and management. In some industrialized nations, use of traditional medicine is equally significant due to cultural and historical influences, as well as preference for complementary therapy. For instance, Canada, France, Germany, and Italy report that between 70-90% of their populations have used traditional medicines under the titles "complementary," "alternative," or "nonconventional." The ongoing use of traditional therapy is not surprising, given that until the middle of the 20th century and the introduction of "modern medication," traditional remedies were the only medicines available.[137,138]

Natural products have been the single most productive source of leads for the development of drugs.[139,140] Since the 1930s, over 180 anticancer drugs have been developed, and more than half of these drugs came from or were based on natural products (Figure 10).[135,140,141]

Anticancer drugs have been established by utilizing natural products in a number of ways: naturally occurring (unmodified), naturally derived (modified), or synthetically mimicked forms.[135,136,140-142] Hence, a number of microorganism-derived compounds have been developed as anticancer drugs, such as anthracyclines (*ie.* doxorubicin and



"N"	Natural product.
"NB"	Natural product "Botanical" (these have been recently approved).
"ND"	Derived from a natural product and is usually a semi-synthetic modification.
"S"	Totally synthetic drug.
S/NM	Synthetic but a competitive inhibitor of an enzyme or receptor (natural product mimic)
"S*"	Made by total synthesis, but the pharmacophore is / was from a natural product.
"S*/NM"	NP pharmacophore that is a competitive inhibitor

Figure 10. Naturally derived compounds in anticancer drug development. From 1930- 2010, over 180 anticancer drugs were developed and nearly half of those drugs were developed from natural products. Depicted in this pie graph are the sources of the drugs. These drugs range from direct natural products to synthetic mimics and derivatives of natural products. Note, less than 25% of the drugs developed had nothing to do with natural products. Figure reproduced from Ref. 140.

daunorubicin), bleomycin, dactinomycin (actinomycin), and mitomycin C. Some of the most beneficial antitumor agents are derived from plants and are categorized based on their relationship to four main compounds: bisindole (vinca) alkaloids, camptothecins, epipodophyllotoxins, and taxanes. In addition, there are several examples of promising natural product-derived antineoplastic agents that are currently in advanced clinical development or have been recently approved from microbial, plant, and marine origin.[136,141]

Natural Products Targeting Topoisomerase II Activity

Naturally Derived Topoisomerase II Poisons Used in the Clinic

A number of topoisomerase II-targeted drugs are derived from natural sources.[57,96] Of the number of clinically relevant antitumor agents derived from plants and microbes (camptothecin, anthracycline and epipodophyllotoxin-based compounds), many of them target topoisomerase activity. These drugs typically function as interfacial poisons, as they inhibit DNA religation and enhance levels of stable topoisomerase-DNA cleavage complexes, ultimately leading to apoptosis.[19,26,36,56,118,120]

One of the first topoisomerase II-targeted agents to be developed was etoposide, which is derived from podophyllotoxin. This natural product is found in *Podophyllum peltatum*, also known as the mayapple or American mandrake plant. Podophyllotoxin has been used as a folk remedy for over a thousand years.[121,125] Although clinical use of podophyllotoxin as an antineoplastic agent was prohibited due to high toxicity, two synthetic analogs, etoposide and teniposide, were developed. These analogs displayed

increased antineoplastic activity and decreased toxicity compared to the parent compound. Importantly, the target changed from tubulin to topoisomerase II when the parent compound was derivatized to these two analogs. [121,125]

In addition, doxorubicin and daunorubicin are produced by the bacterium *Streptomyces peucetius*.[143] Doxorubicin, daunorubicin, and related compounds (idarubicin and epirubicin) are anthracyclines that act as antitumor antibiotics. These anthracyclines are among the most used drugs used in chemotherapy and exert antitumor activity mainly by inhibiting the religation of topoisomerase II-DNA cleavage.[26,47,56,67,144]

Dietary Topoisomerase II Poisons

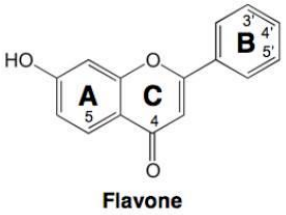
Several naturally occurring dietary compounds act as topoisomerase II poisons.[57,84] These include bioflavonoids (flavones, isoflavones, and flavonols), isothiocyanates, quinones (curcumin oxidation products and thymoquinone), and catechols (epigallocatechin gallate [EGCG]).[58,105,112,145-147] Many of these compounds act as covalent topoisomerase poisons, but some can exert their effects through an interfacial poison mechanism.[57]

Bioflavonoids are a diverse group of polyphenolic compounds that are constituents of many fruits, vegetables, legumes, and plant leaves. Studies suggest that these compounds help protect against cancer, cardiovascular disease, osteoporosis, age-related diseases, and inflammation.[148-153] Although bioflavonoids exert a range of effects on human cells, a number of them are potent topoisomerase II poisons *in vitro* and *in cellulo*.[145,154-157] Notably, most bioflavonoids do not require redox cycling for activity and function primarily by inhibiting enzyme-mediated DNA ligation. Many of the

chemopreventive, cytotoxic, and genotoxic properties of flavones, isoflavones, and flavonols are consistent with their activities as topoisomerase II poisons.[57,145,152,154,155]

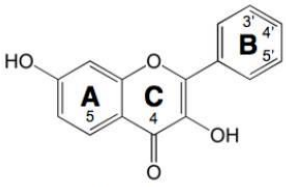
Flavone, isoflavone, and flavonol classes of bioflavonoids are interfacial topoisomerase II poisons (Figure 11).[146,154] In general, structure-function assays showed that the presence of a hydroxyl moiety at the 5- or 4′-position of the bioflavonoids, as in the case of genistein, greatly contributes to the enhancement of enzyme-mediated DNA cleavage.[145,155] Among the bioflavonoids, genistein has the highest activity against the human type II enzymes as an interfacial poison, which was correlated to its cytotoxic effects *in cellulo*.[145,154,158,159] Genistein is one of the most abundant isoflavones in soy. While the main source of genistein is soybeans, other legumes, such as chickpeas, also contain small amounts.[57,141,154-156]

Catechins represent another important class of bioflavonoids (Figure 11).[150,151] Green tea, which is one of the most commonly consumed beverages in the world, is a rich source of catechins and has been suggested to reduce the incidence of breast, prostate, colorectal, and lung cancer in humans. The most abundant catechins in green tea are EGCG and related compounds.[141,146,153,160-164] Although EGCG is a potent topoisomerase II poison, it appears to be a covalent, rather than an interfacial, topoisomerase II poison.[146,147] The mechanistic differences between bioflavonoid classes appear to be related to structural elements in the B and C rings in the compound. While the C-4' hydroxyl of the B-ring is critical for bioflavonoids to act as interfacial topoisomerase II poisons, the inclusion of two additional B-ring hydroxyl groups increases redox activity and is required for compounds to act as covalent topoisomerase II poisons. Because EGCG contains a catechin C-ring, it is unable to act as interfacial topoisomerase II



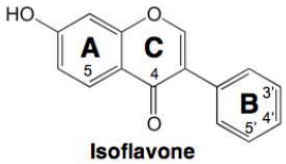
Flavone

Flavones	Position 5	3'	4'	5'	Topo II Activity
Luteolin	OH	OH	OH	H	++
Apigenin	OH	H	OH	H	+
Diosmetin	H	OH	OCH_3	H	+
Chrysin	OH	H	H	H	+/–



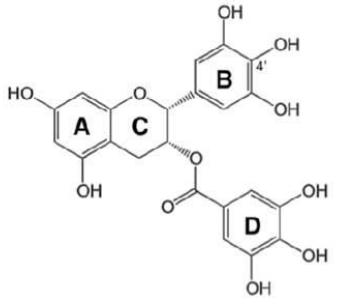
Flavonol

Flavonols	Position 5	3'	4'	5'	Topo II Activity
Myricetin	OH	OH	OH	OH	++
Quercetin	OH	OH	OH	H	++
Kaempferol	OH	H	OH	H	+
Fisetin	H	OH	OH	H	+
Isorhamnetin	OH	OCH_3	OH	H	–
Galangin	OH	H	H	H	–



Isoflavone

Isoflavones	Position 5	3'	4'	5'	Topo II Activity
Genistein	OH	H	OH	H	+++
Daidzein	H	H	OH	H	–
Biochanin A	OH	H	OCH_3	H	–



(–)-Epigallocatechin Gallate (EGCG)

Figure 11. Structures of selected bioflavonoids. Top) Flavones, flavonols, and isoflavones are shown, and the ability of each to enhance topoisomerase II-mediated DNA cleavage is indicated as >8-fold (+++), 6- to 8-fold (++), 3- to 6-fold (+), 2- to 3-fold (+/–), or <2-fold (–) over baseline. Bottom) The structure of the green tea catechin, EGCG, is shown. EGCG has the ability to enhance topoisomerase II-mediated DNA cleavage as well, but through a different mechanism. Adapted from Ref. 145.

poison and functions exclusively as covalent poison.[146,147] Notably, if three hydroxyl groups are included on the B-ring of a flavonol, the compound can act as a dual function topoisomerase II poison that displays both interfacial and redox-dependent characteristics (Figure 11).[57,146,147]

Another source of dietary topoisomerase II poisons can come from glucosinolates, which are sulfur-containing glucosides found in cruciferous vegetables, such as broccoli, cabbage, cauliflower, and kale (Figure 12).[165,166] Many natural isothiocyanates are produced from these glucosinolates and inhibit cell proliferation, display chemopreventive properties, and inhibit tumor growth in xenograft models.[167-169] Isothiocyanates are topoisomerase II poisons *in vitro*.[105] Silencing topoisomerase IIα *in cellulo* decreases DNA damage induced by isothiocyanates. Similar to reactive quinone-based topoisomerase II poisons, isothiocyanates act as covalent poisons and modify several cysteine residues in human topoisomerase IIα. Consistent with the covalent poison mechanism, the ability of isothiocyanates to induce topoisomerase II-mediated DNA cleavage is abolished when the compounds are reduced by prior incubation with excess glutathione.[105]

Additionally, some herbs and spices have been found to act as topoisomerase II poisons (Figure 12). For example, curcumin, which undergoes spontaneous autoxidation, in aqueous solution at a physiological pH, goes through an oxidation intermediate within its oxidation pathway that alters topoisomerase activity. The oxidation intermediate acts as a covalent topoisomerase II poison, while the parent compound and the stable final product have no effect on enzyme activity.[58,170,171] Even in the complex formulation of turmeric, oxidized curcumin intermediates appear to function as topoisomerase II

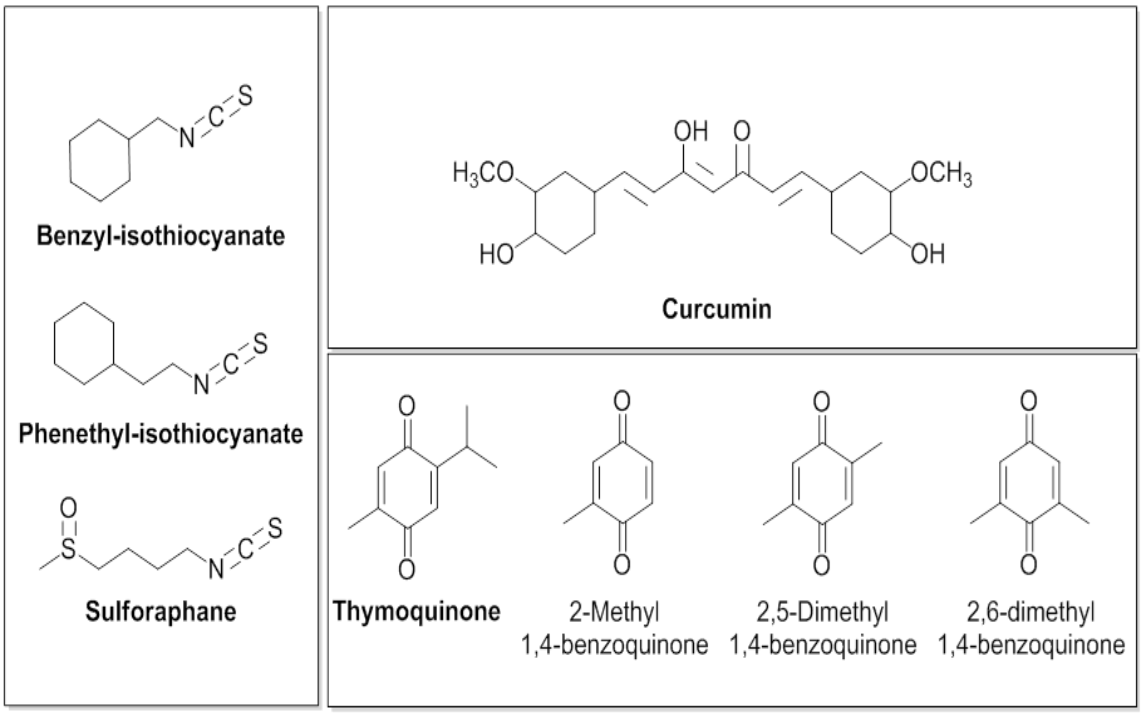


Figure 12. Structures of dietary and related compounds. Left) Structures are shown of sulfur-containing isothiocyanates that are natural products from cruciferous vegetables (broccoli, cabbage, cauliflower, and kale). These isothiocyanates act as covalent poisons and modify several cysteine residues in human topoisomerase IIα. Top) The polyphenol curcumin is shown. Curcumin is the principal flavor and color component of the spice turmeric. An oxidation intermediate of curcumin acts as a covalent poison. Bottom) Thymoquinone is major bioactive compound of black seed and was found to act as a covalent topoisomerase II poison even in its herbal formulation. The similar quinones alongside the thymoquinone structure also enhance topoisomerase II-mediated DNA cleavage. Dietary compounds are bolded.

poisons.[58] The polyphenol curcumin is the principal flavor and color component of the spice turmeric. Beyond its culinary uses, curcumin is believed to positively impact human health and displays antioxidant, anti-inflammatory, antibacterial, and chemopreventive properties.[172-175] Over the years, more than 100 clinical research trials have been initiated to investigate curcumin and its metabolites as a plausible regimented therapy (clinical trials.gov).

Adding to the growing list of dietary and medicinal natural products with activity against human type II topoisomerases, thymoquinone, the major bioactive compound of black seed, was found to act as a covalent topoisomerase II poison even in its herbal formulation.[112] Black seed is a Mediterranean herb with a rich history of medicinal use. Historically, the medicinal herb has been used to treat a variety of illnesses associated with inflammation and displays anticancer activity in cellular and animal models.[176-180] Noticeably, thymoquinone shares structural features with quinone-like covalent topoisomerase II poisons (Figure 12).[57,103,110,112]

All in all, dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the general public due in part to their demonstrated ability to suppress cancers. Over the past 20 years, studies have suggested that diets rich in fruits, vegetables, and spices leads to a lower incidence of cancers (stomach, esophagus, lung, oral cavity and pharynx, endometrium, pancreas, and colon).[93,141,148-152,165,166] Although traditional herbal medicine usage has been stifled since the introduction of modern medicine, researchers and the public are still interested in the wealth of information to be gained from naturally occurring sources for the purposes of

chemopreventative, alternative, and anticancer therapy, as well as for drug discovery and development.

Topoisomerase II-Associated Leukemias and Side Effects

Although topoisomerase II is an important target for chemotherapeutic and chemopreventive agents, there are unfortunate secondary effects that can accompany treatment with topoisomerase II poisons. Evidence suggests that use of some of the frontline therapies used to target topoisomerase II can cause chromosomal translocations that can lead to specific leukemias. About 2-3% of patients treated with etoposide and other topoisomerase II-targeted regimens are eventually diagnosed with treatment-related leukemias such as acute myeloid leukemia (AML).[19,28,48,95,125,181-184] Most of these leukemias are characterized by translocations with breakpoints in the mixed lineage leukemia (*MLL*) gene at chromosomal band 11q23. Several breakpoints in the *MLL* gene have been identified and are located in close proximity to topoisomerase II-DNA cleavage sites that are induced by etoposide.[95,183,185,186]

In addition to treatment-related leukemias, ~80% of infants with AML or acute lymphoblastic leukemia (ALL) display translocations that involve the *MLL* gene. The chromosomal translocations associated with these cancers have been observed *in utero*, indicating that infant leukemias are initiated during gestation.[28,187,188] Epidemiological studies indicate that the risk of developing these infant leukemias increases more than 3-fold when pregnant women consume foods/drinks that are rich in bioflavonoids (*i.e.* soy and green tea) and other naturally occurring topoisomerase II poisons.[28,187-190] Similarly, treatment of cultured human cells with dietary bioflavonoids induces cleavage within the

MLL gene. Compounds that display the highest *in vitro* topoisomerase II-DNA cleavage activity tend to show the greatest propensity to generate breaks in the *MLL* gene in cultured cells.[189] Consequently, the same topoisomerase II activity that is essential for normal cell growth and that is targeted to treat a number of human malignancies can be disadvantageous when targeted by phytochemicals or topoisomerase poisons used in treatment, possibly leading to leukemia through chromosomal translocations.

Moreover, mounting evidence suggests that topoisomerase IIβ is the isoform primarily responsible for initiating at least some topoisomerase II–associated secondary malignancies.[12,28,56,191,192] A recent study demonstrated that a cardiomyocyte specific deletion of topoisomerase IIβ protected mouse hearts from doxorubicin-induced DNA and mitochondrial damage. Since expression of topoisomerase IIα, but not topoisomerase IIβ, is proliferation dependent, differentiated tissues almost exclusively express the β isoform.[28,53] While the precise mechanism by which doxorubicin and other drugs induce cardiac damage remains controversial, research has begun to elucidate the actions of the drug against topoisomerase IIβ as a likely cause of cardiotoxicity.[12,193]

Nonetheless, targeting topoisomerase II has been extremely successful in a variety of settings (*i.e.* anticancer and chemopreventive). Thus, there is still a need to identify new agents that target topoisomerase II, as well as to further understand the detailed mechanism (structure-functions relationships, isoform specificity, and side effects) of currently used topoisomerase II-targeting agents.

Scope of Dissertation

Beyond the critical cellular functions of topoisomerase II, the enzyme is the target for a number of widely prescribed anticancer drugs that are used in the treatment of breast, lung, and prostate cancer, sarcomas, and hematological malignancies. With a broad spectrum of anticancer drugs having emerged directly from natural sources or derived from natural products, it is no surprise that they include a number of compounds that alter topoisomerase II activity.

The dissertation research presented here is focused on characterizing the mechanism by which natural products enhance human topoisomerase IIα-mediated DNA cleavage. The goals of the research presented are to identify naturally derived compounds that alter human topoisomerase II activity and to characterize their mechanism of action. Compounds that were originally extracted from natural sources or were synthesized as a derivative of a natural parent compound were examined for activity against topoisomerase II. These include a library of water-soluble extracts from Mediterranean plants, olive leaf metabolites, ellipticine-based compounds, and quinone-type compounds from the fungus *Septofusidium berolinense*.

Chapter I reviews the fundamentals needed to understand topoisomerase function and purpose, as well as the importance of identifying and understanding new topoisomerase II-targeting drugs and chemopreventatives from natural sources. Chapter II describes the materials and methods used in the research presented in Chapters III-V and the appendix.

Chapter III identifies and characterizes new phytochemicals that enhance topoisomerase II-mediated DNA cleavage from a double-blind screen of a library of 341 Mediterranean plant extracts. Results indicated that an extract from *Phillyrea latifolia* L., a member of the olive tree family, displayed high activity against the human enzyme. Metabolites found in the complex formulation of olive leaves, fruit, and oil enhanced topoisomeraseII-mediated DNA cleavage. Specifically, hydroxytyrosol, oleuropein, and verbascoside displayed hallmark characteristics of covalent topoisomerase II poisons. The potency of these olive metabolites increased 10– to 100–fold in the presence of an oxidant. This research was published in *Biochemistry*.[194]

Chapter IV describes the investigation of two potential topoisomerase II-targeting drugs using two ellipticine-based compounds, ET-1 and ET-2. Through a series of biochemical assays, the new ellipticine derivatives were determined to act as catalytic inhibitors of human topoisomerase IIα and are both more potent than the parent compound, ellipticine. The potency of ET-1 and ET-2 as catalytic inhibitors of topoisomerase IIα appeared to be related to their ability to intercalate into the double helix. This research was published in *Bioorganic & Medicinal Chemistry Letters*.[195]

Chapter V describes the quest to uncover the basis for which two recently identified fungal compounds, GE-1 and GE-2, display cytotoxic activity towards a variety of human cancer cell lines by assessment towards topoisomerase II activity. The quinone compound, GE-2, was characterized as a covalent topoisomerase II poison and enhanced DNA cleavage ~4–fold. In contrast to GE-2, the hydroquinone, GE-1, did not enhance DNA cleavage mediated by topoisomerase IIα. However, the activity and potency of GE-1 was dramatically enhanced under oxidizing conditions. Results from

these studies suggested that topoisomerase IIα may play a role in mediating the cytotoxic effects of these fungal metabolites. This research was published in *Chemical Research in Toxicology.*[196]

Concluding remarks for the research presented in this dissertation are provided in Chapter VI.

In the appendix, research that describes the pursuit to better understand the possible adduction sites of covalent poisons on human topoisomerase IIα is presented. This research was focused on the effects of cysteine to alanine mutations at residues 104, 427, and 427/455 on enzyme activity. Follow-up studies compared His-tagged wild-type to the His-tagged C104A enzyme activity with both the interfacial poison etoposide and the covalent poison hydroxytyrosol. These studies were carried out using site-directed mutagenesis of human topoisomerase IIα, enzyme purification, and activity assays. The appendix also contains the materials and methods prepared by our collaborators.

CHAPTER II

MATERIALS AND METHODS

Materials

Enzymes

Recombinant wild-type human topoisomerase IIα, topoisomerase IIβ, and Top2αΔ1175 [a deletion mutant (residues 1-1175) of human topoisomerase IIα] were expressed from the galactose-inducible YEpWob6 plasmid (12.07kb) in *Saccharomyces cerevisiae* JEL-1Δtop1 and purified as described previously.[197-200] A modified YEpWob6 vector containing the wild-type human topoisomerase IIα gene and a C-terminal c-myc and hexahistidine tag (*6XHis)* [201] was obtained from Dr. Joseph Deweese (Lipscomb University). Recombinant 6XHis- human topoisomerase IIα and mutants were generated, expressed, and purified as indicated in the procedures section below. The catalytic core of human topoisomerase IIα (residues 431-1193) was a gift from J. Deweese and was expressed and purified as described previously.[66,201,202] Enzymes were stored in liquid nitrogen (long-term storage) or at -80 °C as a 1.5 mg/mL stock in 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 750 mM KCl, 5% glycerol. The residual concentration of dithiothreitol was <2 μM in final reaction mixtures. Human topoisomerase I was obtained from Invitrogen.

DNA Substrates

Negatively supercoiled pBR322 DNA was prepared from *Escherichia coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Kinetoplast DNA (kDNA) was isolated from *Crithidia fasciculata* as previously described.100 Relaxed pBR322 plasmid DNA was generated by treatment with topoisomerase I for 30 min as previously described,101 followed by a PCI extraction, ethanol precipitation, and resuspension in 5 mM Tris-HCl (pH 8.5) and 500 **μ**M EDTA. Linear radiolabeled pBR322 was prepared as follows. The pBR322 DNA plasmid was linearized by treatment with *Hin*dIII, and terminal 5'-phosphates were removed and replaced with [^{32}P]phosphate by treatment with calf intestinal alkaline phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled DNA was linearized using *Eco*RI, and the 4330 bp singly-end-[^{32}P] labeled fragment was purified from the short *Eco*RI-*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Reagents

Analytical grade etoposide, DL-dithiothreitol (DTT), adenosine 5′-triphosphate (ATP) disodium salt hydrate, was purchased from Sigma-Aldrich. Etoposide was prepared as a 20 mM stock in 100 % DMSO and stored at room temperature. DTT and ATP were prepared as 500 mM and 20 mM stocks, respectively, and stored at -20 °C. Potassium ferricyanide [$K_3Fe(CN)_6$] was obtained from Acros and was stored at 4 °C as a 50 mM stock solution in deionized purified water. [γ-^{32}P]ATP (5000 μCi) was purchased from Perkin Elmer and stored at -20 °C.

Olive Leaf Metabolites

Analytical grade tyrosol (4-hydroxyphenylethanol), DMPE (3,4-dimethoxyphenylethanol), HMPE (4-hydroxy-3-methoxyphenylethanol), and oleuropein were purchased from Sigma-Aldrich. Analytical grade hydroxytyrosol (3,4-dihydroxyphenylethanol), verbascoside, and caffeic acid (3,4-dihydroxyphenylpropionic acid) were obtained from LKT Laboratories. Tyrosol, hydroxytyrosol, oleuropein, verbascoside, and 4-hydroxy-3-methoxyphenylethanol were prepared as 20 mM stocks in deionized purified water and stored at -20 °C. Caffeic acid, 3,4-dimethoxyphenylethanol, and 4-hydroxy-3-methoxyphenylethanol were prepared as 20 mM stocks in 100 % DMSO and stored at room temperature.

Ellipticine and Derivatives

Analytical grade ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) was purchased from Sigma-Aldrich. The ellipticine derivatives N-methyl-5-demethyl ellipticine (ET-1) and 2-methyl-N-methyl-5-demethyl ellipticinium iodide (ET-2) were synthetically generated as described in the appendix and provided to us by Zeki Topcu. Compounds were prepared as 20 mM stock solutions in 100% DMSO and stored at 4 ºC.

Quinone-like Compounds

Two quinone-type compounds, 3,6-dihydroxy-2-propylbenzaldehyde (GE-1) and 2-hydroxymethyl-3-propylcyclohexa-2,5-diene-1,4-dione (GE-2), were isolated from the ascomycete fungus *S. berolinense* as part of a search for new bioactive secondary

metabolites.[203] GE-1 and GE-2 were provided by Zeki Topcu. Compounds were prepared as 50 mM stock solutions in 100% DMSO and stored at 4 ºC.

Plant Extracts

A library of methanol/water extracts from 341 native Mediterranean plants, mainly from arid lands, the Tel Aviv University Botanical Garden and traditional Bedouin medicinal plants.[204-206] Based on results with the original library, a second library of 36 extracts from the leaf, bark, flowers, or fruit of 11 individual olive tree species was established.

Plant extracts were prepared as described by Kaiser et al.[207] The 341 plants extracts were resuspended in deionized purified water at a final concentration of 2 mg/mL and screened in a blind fashion. Samples from the olive tree extract library were prepared as described above, resuspended to a final concentration of 10 or 20 mg/mL in deionized purified water, and stored at -20 °C.

Commercial Extracts

Commercial olive leaf extract (Olive Leaf Plus™, ~30% oleuropein) was purchased from Life-Flo and prepared as a 20 mg/mL stock in deionized purified water. The manufacturer did not identify the species of olive leaves used in this preparation. Extra virgin olive oils, pressed from the indicated subspecies of *O. europea*, including Olive Oil Store Ultra Arbosana (arbosana olives), Olive Oil Store Ultra Koroneiki (koroneiki olives), and Lucini Select (a mixture of frantoio, moraiolo, leccino, maurino, and pendolino olives), were stored at room temperature. Soluble extra virgin olive oil

extracts were prepared by vigorously vortexing a 1:1 mixture of oil and deionized purified water for 5 min, using 30 s pulses. The oil and water phases were separated by centrifugation at 8,000 x g for 10 min, at room temperature, and the aqueous phase was used for subsequent experiments.

Procedures

Plasmid DNA Cleavage

DNA cleavage reactions were performed as described by Fortune and Osheroff.[208] Reaction mixtures contained 110 nM human topoisomerase IIα or 430 nM topoisomerase IIα catalytic core and 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer [10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol]. DNA cleavage reaction mixtures were incubated at 37 °C for 6 min, and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the type II enzyme. Reaction samples were mixed with 2 μL of agarose loading dye [60% sucrose in 10 mM Tris-HCl pH 7.9, 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 2 min, and subjected to electrophoresis using 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized by UV light and quantified using an Alpha Innotech digital imaging system. Double-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid to linear molecules. In some cases, EcoRI was used as a control for

double-strand cleavage (100%) and etoposide was used as positive control as an interfacial topoisomerase IIα poison.

Enzyme-linked DNA Cleavage Controls

DNA cleavage reactions were carried out in the presence of 0–1000 μM GE-1 or GE-2 or 100 μM etoposide. Unless stated otherwise, compounds were added last to reaction mixtures. In reactions that determined whether DNA cleavage by human topoisomerase IIα was reversible, 2 μL of 250 mM EDTA was added to samples prior to treatment with SDS. To determine whether cleaved DNA was protein-linked, proteinase K treatment was omitted.

Oxidation-Reduction DNA Cleavage

To determine whether cleaved DNA was protein-linked, proteinase K treatment was omitted. To examine the effects of a reducing agent (DTT) or oxidizing agent [$(K_3Fe(CN)_6$] on the actions of 250 μM GE-1 or GE-2 against topoisomerase IIα, 250 μM DTT or 50 μM $K_3Fe(CN)_6$ (final concentration) was incubated with the compounds for 10 min before their addition to DNA cleavage reaction mixtures.

DNA Cleavage Site Utilization

DNA cleavage sites were mapped using the procedure of Hawtin et al.[209] pBR322 DNA was linearized by treatment with *Hin*dIII, and terminal 5'-phosphates were removed and replaced with [^{32}P]phosphate by treatment with calf intestinal alkaline phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled DNA

was digested using *Eco*RI, and the 4330 bp singly-end-[^{32}P] labeled fragment was purified from the short *Eco*RI-*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Reaction mixtures contained 4 nM [^{32}P]-labeled DNA substrate and 44 nM human topoisomerase IIα in 50 µL of DNA cleavage buffer. Assays were carried out in the absence of compound, or in the presence of 10 µM etoposide or 0-1000 µM GE-1 or GE-2. Reactions were initiated by the addition of the topoisomerase IIα and were incubated at 37 °C for 1 min. DNA cleavage intermediates were trapped by addition of 5 µL of 5% SDS, followed by 3.75 µL of 250 mM EDTA (pH 8.0). Proteinase K (5 µL of a 0.8 mg/mL solution) was added and incubated at 45 °C for 30 min to digest the topoisomerase IIα. DNA products were precipitated in 100% ethanol and 3 M NaOAc, washed in 70% ethanol, dried, and resuspended in 6 µL of 40% formamide, 10 mM NaOH, 0.02% xylene cyanol FF, and 0.02% bromophenol blue. Samples were subjected to electrophoresis in a 6% denaturing polyacrylamide-sequencing gel in 100 mM Tris-borate (pH 8.3), and 2 mM EDTA. The gel was dried and exposed to an imaging screen (Bio-Rad). [^{32}P]-labeled DNA cleavage products were analyzed on a Pharos Molecular Imager FX (Bio-Rad).

Persistence of Cleavage Complexes

The persistence of topoisomerase IIα-DNA cleavage complexes was determined using the procedure of Gentry et al.[210] Initial reactions contained 50 nM DNA and 550 nM topoisomerase IIα in a total of 20 µL of DNA cleavage buffer. Reactions were carried out in the absence or presence of 250 µM GE-2. Reactions were incubated at 37 ºC for 6

min and then diluted 20–fold with 37 ºC DNA cleavage buffer. Aliquots (20 µL) were removed at times ranging from 0–24 h, and DNA cleavage was stopped by the addition of 2 µL of 5% SDS followed by 2 µL of 250 mM EDTA (pH 8.0). Samples were processed as described above for plasmid cleavage assays. The persistence of cleavage complexes was monitored by the maintenance of the linear reaction product over time.

Decatenation of Kinetoplast DNA

Decatenation assays were carried out using the procedure of Miller *et al.*[211] Reaction mixtures contained 0.2 µg of kinetoplast DNA (kDNA) from *Crithidia fasciculate* and 75 nM human topoisomerase IIα in a final volume of 20 µL of 50 mM Tris-Cl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol.[212] Reactions were carried out in the presence of 0-5 mM ellipticine, ET-1, or ET-2. Mixtures were incubated for 15 min at 37 °C and terminated with 5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol. Samples were mixed with 2 µL of agarose loading dye [60% sucrose in 10 mM Tris-HCl pH 7.9, 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated for 2 min at 45 °C, and subjected to electrophoresis using 1% agarose gels in 40 mM Tris-borate, pH 8.3 and 2 mM EDTA containing 0.5 µg/mL ethidium bromide. DNA bands were visualized by UV light. DNA decatenation was monitored by the conversion of large catenated networks that remained at the origin to minicircles.

DNA Intercalation

DNA intercalation reactions were carried out using the protocol of Fortune *et al.*[213] Human DNA topoisomerase I (0.5 U) and 300 ng of relaxed pBR322 were

incubated in 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 0.02 mM EDTA, 0.1 mM dithiothreitol, and 6 μg/ml bovine serum albumin in a final volume of 20 μL. Reactions were carried out in the presence of 0-25 μM ellipticine, ET-1, or ET-2. Ethidium bromide (10 μM), a well-characterized intercalator, was used as positive control, and etoposide (250 μM), a non-intercalative topoisomerase II poison, was used as a negative control. Mixtures were incubated for 15 min at 37 °C, and reactions were stopped by the addition of 3 μL of stop solution (0.77% SDS 77.5 mM EDTA, pH 8.0). Samples were extracted using 20 μL of phenol:chloroform:isoamyl alcohol (25:24:1), and the aqueous layer was mixed with 2 μL of agarose loading dye and heated for 5 min at 45 °C. Intercalation products were subjected to electrophoresis in a 1% agarose gel in 100 mM Tris-borate and 2 mM EDTA. Gels were stained for 30 min using 0.5 μg/mL ethidium bromide, rinsed in deionized water, and DNA bands were visualized as described above. DNA intercalation was monitored by the conversion of relaxed to supercoiled plasmid molecules.

Binding of Plasmid DNA

DNA binding reactions were carried out using the procedure of Osheroff.[214] Reaction mixtures contained 10 nM negatively supercoiled pBR322 DNA and 0-550 nM human topoisomerase IIα in a final volume of 20 μL of 10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol. Mixtures were incubated for 6 min at 37 °C in the absence or presence of 25 μM ET-1 or 25 μM ET-2. Enzyme-DNA samples were mixed with 2 μL of agarose loading dye and subjected to electrophoresis using 1% agarose gels in 40 mM Tris-acetate, pH 8.3 and 2 mM EDTA.

Gels were stained for 30 min using 0.5 μg/mL ethidium bromide, rinsed in deionized water, and DNA was visualized as described above. Enzyme-DNA binding was monitored by the shift of the DNA from the position of negatively supercoiled [(-)SC] plasmid to the origin.

Covalent Adduction

Reaction mixtures contained 110 nM human topoisomerase IIα in buffer DNA cleavage buffer. Assays were carried out in the absence of compound, or in the presence of 10 μM hydroxytyrosol plus 10 μM $K_3Fe(CN)_6$. Reactions were incubated at 4 °C for 5 min, followed by an incubation at 37 °C for 5 min. The samples were then acid precipitated using trichloroacetic acid (TCA) precipitation with an acetone wash in preparation for LC MS/MS. [215] Samples were precipitated by adding TCA to a final concentration of 25% and incubating at -20 °C for 20 min. Samples were centrifuged at 13000 rpm for 20 min and the supernatant was aspirated. Samples were washed in ice-cold 95% acetone (twice) by addition of 95% acetone, gently vortexing, and incubation at -20 °C for 20 min. Samples were air-dried and stored at -80 °C until processed. The samples were processed by the Vanderbilt MSRC proteomics laboratory using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry. Mass spectrometry results were analyzed using the Skyline 3.5 program. (XXX)

Site-directed Mutagenesis

Using the modified YEpWob6 vector containing the wild-type human topoisomerase IIα gene and a C-terminal c-myc and *6XHis* tag, mutations in the 6XHis-

tagged gene were generated by using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent).[201] The primer pairs listed below were used as templates to mutate human topoisomerase II at the sites: C427A, C455A, C427A/C455A (Dbl mt), and C104A

C427A_Fwd 5'-gcccaagtccagttaaacaagaaggcttcagctgtaaaacataatagaat

C427A_Rev 5'-attctattatgttttacagctgaagccttcttgtttaactggacttgggc

C455A_Fwd 5'-gccgaaactccactgaggctacgcttatcctgactg

C455A_Rev 5'-cagtcaggataagcgtagcctcagtggagtttcggc

C104A_Fwd 5'-acaaagggacccaaaaatgtctgctattagagtcacaattgatccg

C104A_Rev 5'-cggatcaattgtgactctaatagcagacatttttgggtccctttgt

The mutated positions are underlined. The mutated genes encoding the C427A and C455A mutations were sequenced for accuracy using the primer 5'-cccaagagctttggatcaac. The mutated gene encoding the C104A mutation was sequenced for accuracy using the primer 5'-gtggaattagtgacccagcaa.

Expression of Human Topoisomerase IIα and Mutants

Human topoisomerase IIα wild-type, mutant C427A, C455A, C427A/C455A, or C104A DNA was transformed into yeast cells and expressed as previously described.[197] Briefly, the cells were grown at 30 °C, shaking, in ura$^-$ minimal medium, supplemented with 2% (w/v) glucose, 3% (v/v) glycerol, and 2% (w/v) lactic acid. At log phase the culture was diluted 100-fold into the same medium without glucose. Cells were induced at an OD_{600} of 0.3-0.4 with galactose (2% (w/v) final concentration). Cells were harvested after 24 h, washed with autoclaved water, and then with lysis buffer [50 mM Tris-HCl (pH 7.7), 10% (w/v) glycerol, 1 mM EDTA, 1 mM EGTA] plus 25 mM NaF, 1

mM $Na_2S_2O_5$, 1 mM BME and 1 mM PMSF]. Cells were then resuspended in 2 ml of lysis buffer/g of wet-packed cells, frozen rapidly in liquid nitrogen, and stored at -80 °C.

Purification of 6XHis-Human Topoisomerase IIα and Mutants

In preparation for protein purification, the harvested cells were quickly thawed under running lukewarm water and lysed using Y-PER Plus (Thermo-Scientific) at 2 ml/g containing 500 mM NaCl, 1 mM PMSF and protease inhibitors (1 µg/ml leupeptin and 1 µg/ml pepstatin A), while shaking at RT for 15 min. Unless otherwise noted, the following purification steps were carried out at 4 °C. The lysate was cleared by centrifugation at 14 000 rpm using a JA 25.50 rotor for 10 min. Imidazole was then added to the cleared lysate to the final concentration of 5 mM and the lysate was subjected to batch binding to 2 mL of Ni-NTA agarose beads (Qiagen) slurry for 1 hr. After binding, the resin was harvested at 700 rpm in a Sorvall H1000B rotor (Dupoint Instruments) for 3 min. The nickel resin was then washed in 14 ml of wash buffer 1 [20 mM Tris-HCl (pH 7.9), 1 M NaCl, 30 mM imidazole, 1 mM BME, 1 mM PMSF, and protease inhibitors] for 10 min followed by 30 ml of wash buffer 2 [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 30 mM imidazole, 1 mM BME, 1 mM PMSF, and protease inhibitors] for 10 min. 5 ml of wash buffer 2 was added to the protein bound-nickel resin and transferred into a 1 x 10 cm Econo column (BioRad), while the extra wash buffer was removed by gravity. The enzyme was eluted by gravity with 5 mL of elution buffer [20 mM Tris-HCl (pH 7.9), 0.5 mM NaCl, 300 mM imidazole, 1 mM BME, 1 mM PMSF, and protease inhibitors]. 1 mL fractions were collected and assessed for protein by SDS-PAGE and fractions 1-4 were pooled after protein presence verification.

The protein was further purified and concentrated by a P11 phosphocellulose (P-cell) column (Whatman) equilibrated in P-200 buffer [50 mM Tris-HCl (pH 7.7), 200 mM KCl, 40% (w/v) glycerol, 0.1 mM EDTA, 0.5 mM DTT]. The protein collected from the eluted fractions was diluted with P200 buffer until the conductivity of the sample buffer was below that of the P-cell buffer. The protein sample was then loaded onto a 1 ml P-cell column, washed with 1 column volume of P200 buffer and eluted by gravity with 3 column volumes of P750 buffer [50 mM Tris-HCl (pH 7.7), 750 mM KCl, 40% (w/v) glycerol, 0.1 mM EDTA, 0.5 mM DTT] in 3 drop fractions. The fractions were the assessed for protein presence by Bradford analysis and fractions containing protein were pooled.

Bradford analysis was used to determine the protein concentrations. A 50 kDa MWCO Amicon Ultra concentrator was used to further concentrate the protein at 2500 rpm in a Sorvall H1000B rotor set at 4 °C. Homogeneity of the topoisomerase II preparations was determined by SDS-polyacrylamide gel analysis of a Mini-Protean TGX 7.5% polyacrylamide gel (Bio-Rad) after staining with Coomassie dye. Fractions containing topoisomerase II enzyme were pooled and stored in liquid nitrogen for later use. Wild-type His-tagged topoisomerase IIα was prepared parallel to the mutant enzymes.

Enzyme activity

DNA cleavage reactions were performed similar to the plasmid cleavage methods provided above. Reaction mixtures contained 0-300 nM His-tagged human topoisomerase IIα and mutants and 10 nM negatively supercoiled pBR322 DNA in a total of 20 µL of

cleavage buffer. DNA cleavage reaction mixtures were incubated at 37 °C for 6 min, and the enzyme-DNA cleavage complexes were stopped, processed, and visualized as described above for plasmid cleavage assays.

DNA relaxation assays were based on the procedure of Fortune and Osheroff. [208] Reaction mixtures contained 0.02-110 nM enzyme, 5 nM negatively supercoiled pBR322 DNA and 1 mM ATP in a total of 20 µl of relaxation buffer (10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 175 mM KCl, 5 mM MgCl2, and 2.5% glycerol). Samples were incubated at 37 °C for 15 min, and DNA relaxation was terminated by the addition of 3 µl of stop solution [0.77% SDS, 77 mM EDTA (pH 8.0)]. Samples were mixed with 2 µl of agarose gel loading buffer [60% sucrose in 10 mM Tris-HCl (pH 7.9)], heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris borate (pH 8.3), 2 mM EDTA. The gel was stained for 30 min with gentle shaking in 200 ml of TBE containing 1 µg/ml EtBr and rinsed in deionized water. DNA bands were visualized with ultraviolet light. DNA relaxation activity was monitored by the conversion of negatively supercoiled DNA to relaxed DNA. His-tagged human topoisomerase IIα was used as an enzyme control.

CHAPTER III

EFFECTS OF OLIVE METABOLITES ON DNA CLEAVAGE MEDIATED BY HUMAN TYPE II TOPOISOMERASES

Introduction

A broad spectrum of anticancer drugs comes directly from natural sources or is derived from natural products. Many of these compounds are botanical in nature. Plants have been used for culinary and medicinal purposes for millennia. The Mediterranean basin is home to a biologically diverse plant biome and is a particularly rich source of botanicals with medicinal properties. [93,216,217] Many of these have been used in traditional Bedouin and Israeli medicine since antiquity. Phytochemicals derived from Mediterranean plant species have been shown to provide a variety of health benefits and display anti-inflammatory, anticancer, cardioprotective, and chemopreventative properties.[93,216,217]

In an effort to discover novel phytochemicals with activity against human type II topoisomerases, we carried out a blind screen of a library of 341 Mediterranean plant extracts to determine whether any of them increased levels of DNA cleavage mediated by topoisomerase IIα. Species in the library were primarily from arid lands or the Tel Aviv University Botanical Garden and included plants used in traditional Bedouin

medicine.[204,205] An extract from *Phillyrea latifolia* L., a member of the *Oleaceae* family of olive trees, displayed high activity against the human enzyme. Using a metabolomics approach, we identified several polyphenols as potential candidates for topoisomerase II poisons. From these compounds, we determined that hydroxytyrosol, oleuropein, and verbascoside were covalent poisons. The activities of these compounds were accentuated under oxidizing conditions. Finally, commercial olive leaf extract and extra virgin olive oils also poisoned human topoisomerase IIα.

Results and Discussion

An Extract of P. latifolia L. Enhances DNA Cleavage Mediated by Human Topoisomerase IIα.

A number of dietary polyphenols and isothiocyanates with chemopreventive or anticancer properties have been found to be topoisomerase II poisons.[57,58,105,112,145-147,154,164,218] In all of the above cases, studies were initiated by examining the effects of purified compounds on the activity of the type II enzyme. In order to take a broader and less biased approach to the discovery of naturally occurring topoisomerase II poisons, a library of 341 Mediterranean plant extracts was screened for its effects on DNA cleavage mediated by human topoisomerase IIα. Extract #263 prepared from the leaves of *P. latifolia* L., a member of the *Oleaceae* olive tree family, displayed high activity against topoisomerase IIα and increased levels of DNA cleavage nearly 8–fold at 200 μg/mL (Figure 13).

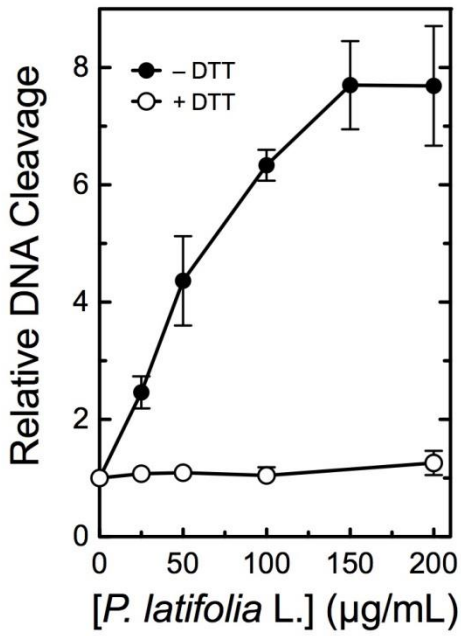


Figure 13. Effects of *P. latifolia* L. on DNA cleavage activities mediated by human topoisomerase II. The effects of plant extract #263 (*P. latifolia* L.) on the cleavage of negatively supercoiled DNA by topoisomerase IIα were determined in the absence (filled circles) or presence (open circles) of 100 µM DTT. DNA cleavage levels were calculated relative to a control reaction that contained no extract. Error bars represent the standard deviation of at least three independent experiments.

Previous metabolomic studies on *P. latifolia* L. leaves indicate the presence of several bioflavonoid derivatives, including glucosides and rutinosides of apigenin, quercetin, and luteolin.[219,220] Although the three unmodified bioflavonoids are known interfacial topoisomerase II poisons, it is not known whether the above glycoside derivatives retain activity within the soluble extract.[146,154] Therefore, to determine whether these bioflavonoid glycosides (or other potential interfacial poisons) represented the topoisomerase II-active compounds in *P. latifolia* L., the extract was assayed for activity in the presence of a reducing agent, dithiothreitol (DTT). As seen in Figure 13, the extract lost its activity under reducing conditions. This finding suggests that the active metabolites present in the *P. latifolia* L. extract are covalent poisons, rather than bioflavonoid-based interfacial poisons.[103]

Olive Metabolites Poison Human Type II Topoisomerases

Several phenolic compounds with antioxidant activity are abundant in *P. latifolia* L. and have the potential to act as covalent topoisomerase II poisons.[57,219,220] Among these metabolites are hydroxytyrosol, oleuropein, verbascoside, tyrosol, and caffeic acid. Oleuropein contains an esterified hydroxytyrosol component and verbascoside contains esterified hydroxytyrosol and caffeic acid components. Tyrosol is a breakdown product of hydroxytyrosol. The structures of these compounds are shown in Figure 14.

To determine whether any of the above *P. latifolia* L. metabolites contributed to the activity of the extract against human topoisomerase IIα, the ability of individual compounds to enhance enzyme-mediated DNA cleavage was examined (Figure 15). Hydroxytyrosol, oleuropein, and verbascoside all increased levels of DNA cleavage more

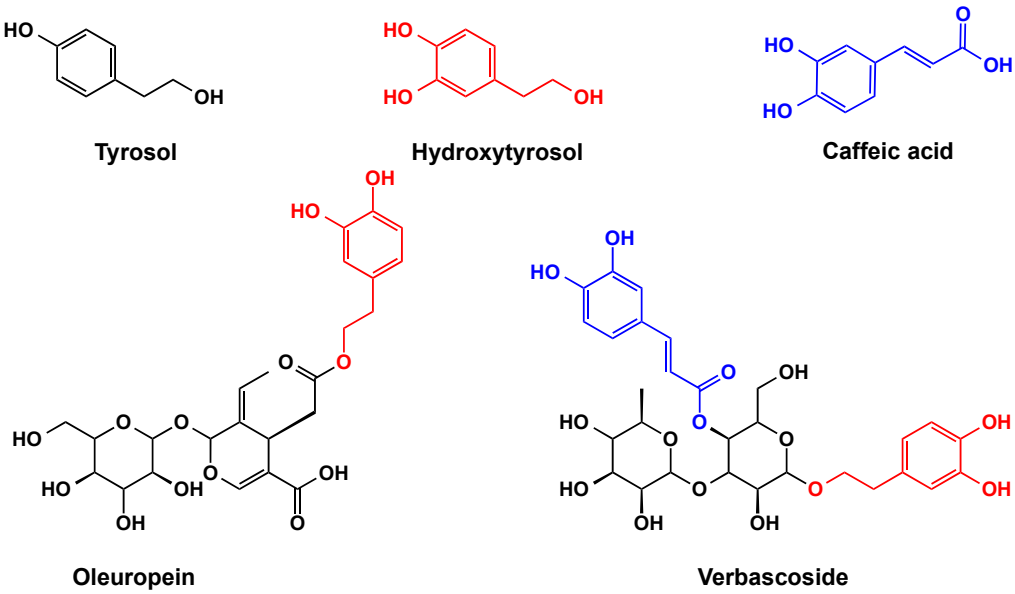


Figure 14. Structures of olive metabolites. Polyphenols present in *P. latifolia* L. and other olive species, including hydroxytyrosol, oleuropein, verbascoside, caffeic acid, and tyrosol are shown. Hydroxytyrosol (red) is a component of oleuropein and verbascoside, and caffeic acid (blue) is a component of verbascoside.

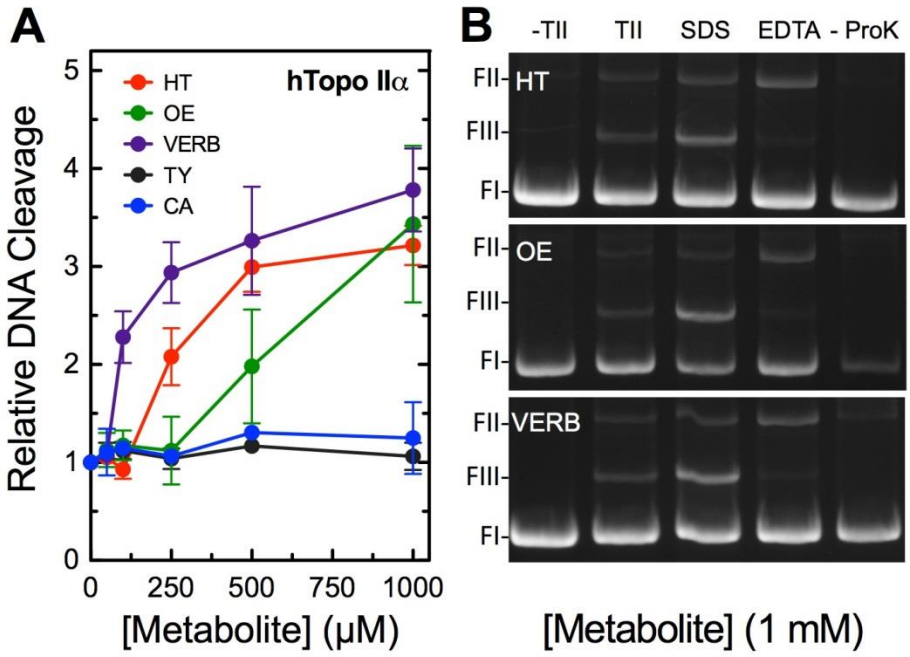


Figure 15. Effects of olive metabolites on DNA cleavage activities mediated by human topoisomerase II. A) The effects of the hydroxytyrosol (HT; red), oleuropein (OE; green), and verbascoside (VERB; purple), caffeic acid (CA; blue), and tyrosol (TY; black) on DNA cleavage mediated by topoisomerase IIα are shown. DNA cleavage levels were calculated relative to a control reaction that contained no metabolite. Error bars represent standard deviations for three independent experiments. B) DNA cleavage induced by hydroxytyrosol (top), oleuropein (middle), or verbascoside (bottom) is reversible and protein-linked. Ethidium bromide-stained agarose gels are shown. Assay mixtures contained DNA with olive metabolites in the absence of enzyme (-TII), topoisomerase IIα with DNA in the absence of olive metabolites (TII), or complete reactions stopped with SDS prior to the addition of EDTA (SDS). To determine whether the reaction was reversible, EDTA was added prior to SDS (EDTA). To determine whether the cleaved DNA was protein-linked, proteinase K treatment was omitted (-ProK). The mobility of negatively supercoiled DNA (form I; FI), nicked circular plasmid (form II; FII), and linear molecules (form III; FIII) are indicated. Gels are representative of three independent experiments.

than 3–fold. Conversely, tyrosol and caffeic acid displayed virtually no activity against the human type II enzyme.

To ensure that the observed DNA cleavage enhancement was mediated by the type II enzyme, several control experiments were performed (Figure 15B). No DNA scission was seen in the presence of hydroxytyrosol (top), oleuropein (middle), or verbascoside (bottom) when the type II enzyme was left out of reactions. Furthermore, enzyme-mediated DNA cleavage induced by the olive metabolites was reversed when the active site Mg^{2+} ions were chelated with EDTA prior to trapping cleavage complexes with SDS. This reversibility is not consistent with an enzyme-independent reaction. Finally, cleaved plasmid products were covalently linked to topoisomerase II. In the absence of proteinase K the linear DNA band disappeared and was replaced by a band that remained at the origin of the gel. These results demonstrate that the DNA cleavage observed in the presence of the metabolites is mediated by topoisomerase IIα.

Although hydroxytyrosol, oleuropein, and verbascoside are all polyphenols, it is likely that they would have to cycle through a quinone form in order to become reactive toward topoisomerase IIα.[57,221] A previous study found that the buffer used for topoisomerase II-mediated DNA cleavage reactions does not readily support redox cycling.[58] This is consistent with the high concentrations of metabolites required to enhance DNA cleavage. Therefore, the effect of an oxidant, $K_3Fe(CN)_6$, on the activity of hydroxytyrosol, oleuropein, and verbascoside toward topoisomerase IIα was examined (Figure 16A). This oxidant has little effect on levels of baseline DNA cleavage mediated by topoisomerase IIα, but had a dramatic effect on the activity of the metabolites. DNA cleavage induced by the metabolites plateaued at 10 μM $K_3Fe(CN)_6$ (Figure 16A, left).

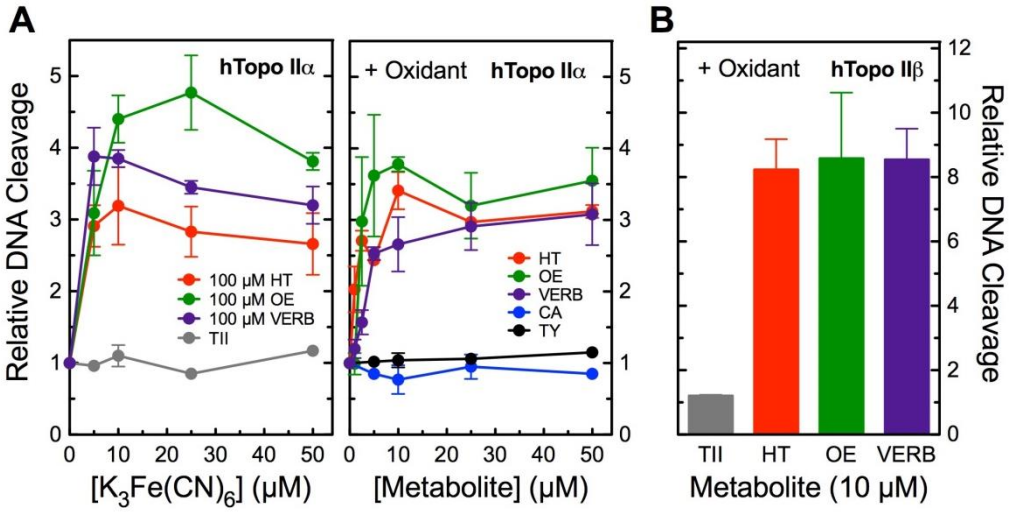


Figure 16. Effects of oxidant on human topoisomerase II-mediated DNA cleavage induced by olive metabolites. A) The effects of an oxidant, $K_3Fe(CN)_6$, on DNA cleavage mediated by topoisomerase IIα were determined in the presence of 100 μM olive metabolites [hydroxytyrosol (HT; red), oleuropein (OE; green), verbascoside (VERB; purple), caffeic acid (CA; blue), and tyrosol (TY; black)] or in the absence of metabolite (TII; gray) **(**left**)**. The effects of olive leaf metabolites on DNA cleavage were determined in the presence of 10 μM $K_3Fe(CN)_6$ **(**right**)**. B) The effects of olive metabolites on DNA cleavage mediated by human topoisomerase IIβ were determined in the presence of 10 μM $K_3Fe(CN)_6$. DNA cleavage levels were calculated relative to a control reaction that contained no metabolite and no oxidant. Error bars represent standard deviations for three independent experiments.

Consequently, this concentration was used for all subsequent reactions that contained the oxidant.

The presence of $K_3Fe(CN)_6$ increased the potency of hydroxytyrosol, oleuropein, and verbascoside toward topoisomerase IIα as much as 100–fold (Figure 16A, right). Whereas 250 μM to 1 mM metabolite was required to increase DNA cleavage between 3– to 4–fold in the absence of oxidant (see Figure 15A), a similar DNA cleavage increase in reactions that contained $K_3Fe(CN)_6$ required only 5-10 μM metabolite. Despite the presence of the oxidant, neither tyrosol nor caffeic acid displayed any significant ability to poison topoisomerase IIα.

The effects of hydroxytyrosol, oleuropein, and verbascoside on DNA cleavage mediated by human topoisomerase IIβ also were assessed (Figure 16B). In the presence of oxidant, all three polyphenols were potent topoisomerase II poisons and increased DNA cleavage >8–fold at a metabolite concentration of 10 μM. The higher relative activity of olive metabolites against topoisomerase IIβ as compared to IIα primarily reflects lower baseline levels of DNA cleavage observed with the β isoform in the absence of poisons.

The effects of hydroxytyrosol, oleuropein, and verbascoside (10 μM) on DNA cleavage site utilization by human topoisomerase IIα were determined in the presence of oxidant (Figure 17). Similar sites of cleavage were induced by all three metabolites. Several of the sites induced by the metabolite were the same as those induced by etoposide, although some sites were utilized to a different extent. Distinct sites also were observed in the presence of the metabolites compared to the drug. Similar DNA cleavage

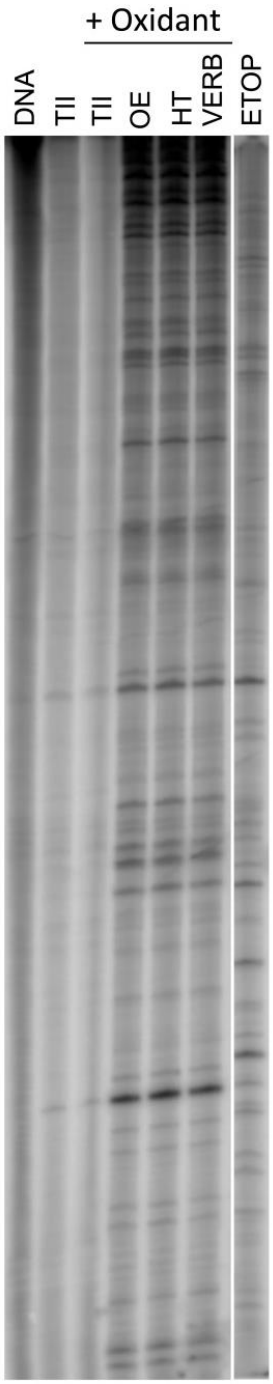


Figure 17. Effects of olive metabolites on DNA cleavage site utilization by human topoisomerase IIα. An autoradiogram of a polyacrylamide gel is shown. Reaction mixtures contained no enzyme (DNA), enzyme in the absence of metabolite (TII), or enzyme in the presence of 10 μM hydroxytyrosol (HT), oleuropein (OE), or verbascoside (VERB) in the presence of 10 μM $K_3Fe(CN)_6$. A control DNA cleavage reaction that contained 20 μM etoposide also is shown. The autoradiogram is representative of three independent experiments.

maps were generated in the presence of 1 mM metabolites in the absence of oxidant (data not shown).

Although etoposide and other interfacial poisons increase topoisomerase II-mediated DNA scission primarily by inhibiting the ability of the enzyme to ligate cleaved molecules, covalent poisons often induce DNA cleavage without displaying large effects on rates of ligation.[19,57,99,103] As seen in Figure 18A, hydroxytyrosol, oleuropein, and verbascoside had relatively little effect on DNA ligation mediated by topoisomerase IIα in the absence or presence of oxidant. In contrast, no ligation was observed in the presence of etoposide. These findings are consistent with the olive metabolites acting as covalent poisons and suggest that they may increase levels of DNA cleavage complexes primarily by enhancing the forward rates of DNA cleavage.

Because covalent poisons adduct topoisomerase II, they cannot dissociate from the enzyme.[19,57,84] Thus, once DNA cleavage complexes are formed in the presence of covalent poisons, they can remain intact for hours.[112] To address the stability of cleavage complexes formed in the presence of hydroxytyrosol, oleuropein, or verbascoside, DNA cleavage complexes were diluted 20–fold and their decay was monitored (Figure 18B). In the absence of poisons, topoisomerase IIα-DNA cleavage complexes undergo a rapid decay and display a half-life less than 1 min.[112] In contrast, cleavage complexes formed in the presence of 10 μM metabolite + oxidant or 1 mM metabolite without oxidant were extremely stable and remained intact for at least 24 h.

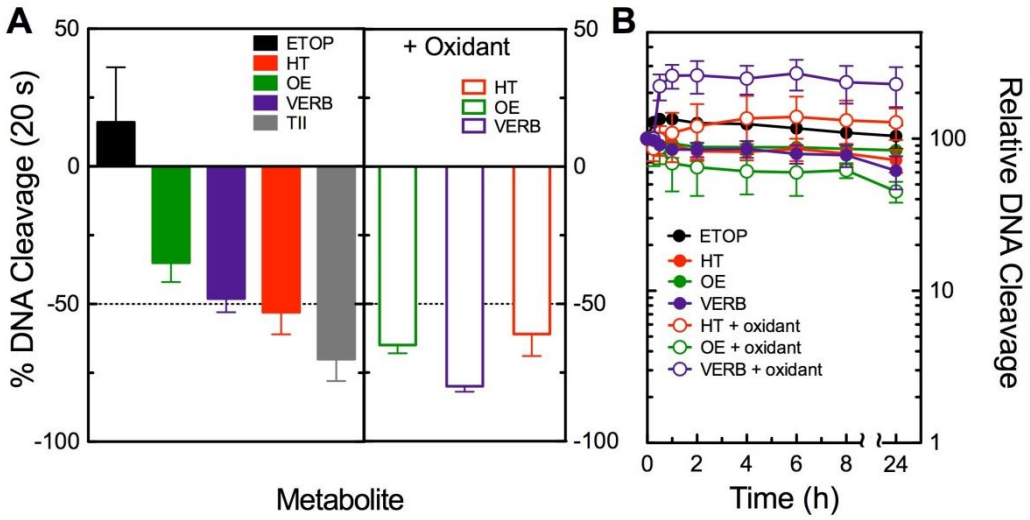


Figure 18. Effects of olive leaf metabolites on human topoisomerase IIα-mediated DNA cleavage complex stability. A) The ability of human topoisomerase IIα to ligate cleaved DNA is shown. Reactions (20 s) were carried out in the presence of no metabolite (TII; gray), hydroxytyrosol (HT; red), oleuropein (OE; green), verbascoside (VERB; purple) or 100 µM etoposide (ETOP; black). Reactions contained 1 mM metabolite and no oxidant (left) or 10 µM metabolite in the presence of 10 µM $K_3Fe(CN)_6$ (right). B) The effects of olive metabolites on the persistence of topoisomerase IIα-DNA cleavage complexes in the absence or presence of oxidant are shown. Assays were carried out in the presence of 1 mM metabolite (open circles) or 10 µM metabolite + 10 µM $K_3Fe(CN)_6$ (closed circles). Colors are as described above. For both the ligation and persistence reactions, DNA cleavage levels at time zero were set to 100% to allow a direct comparison. Error bars represent the standard deviation of at least three independent experiments.

Hydroxytyrosol, Oleuropein, and Verbascoside are Covalent Topoisomerase IIα Poisons.

The above results, together with the finding that *P. latifolia* L. extracts lost their ability to poison topoisomerase IIα in the presence of a reducing agent, strongly suggest that hydroxytyrosol, oleuropein, and verbascoside are covalent topoisomerase II poisons. Therefore, a series of experiments was carried out in order to address the basis for the actions of the olive metabolites against topoisomerase IIα.

First, if the olive metabolites are covalent poisons, their ability to cycle through an activated quinone form should be critical to their activity.[57,58,147] Therefore, in order to inhibit redox cycling, 1 mM hydroxytyrosol, oleuropein, and verbascoside were incubated with 100 μM DTT prior to their addition to DNA cleavage reaction mixtures. As seen in Figure 19, treatment with the reducing agent abrogated the activity of the olive metabolites (open bars). Levels of DNA scission were reduced to baseline cleavage generated by the type II enzyme.

Second, once covalent poisons have adducted topoisomerase II, their redox state no longer affects their activity.[19,57,103,104] Thus, the addition of reducing agents to reaction mixtures after DNA cleavage−ligation equilibria have been established in the presence of a covalent poison should not reverse the cleavage enhancement. As seen in Figure 19 (stippled bars), the addition of DTT after cleavage complexes had been formed in the presence of hydroxytyrosol, oleuropein, or verbascoside had no significant effect on levels of DNA scission mediated by the type II enzyme.

Third, to further examine the requirement for the conversion of olive metabolites to an activated quinone, one or both of the hydroxyl moieties of hydroxytyrosol were converted to methoxyl groups (4-hydroxy-3-methoxyphenylethanol and 3,4-dimethoxy-

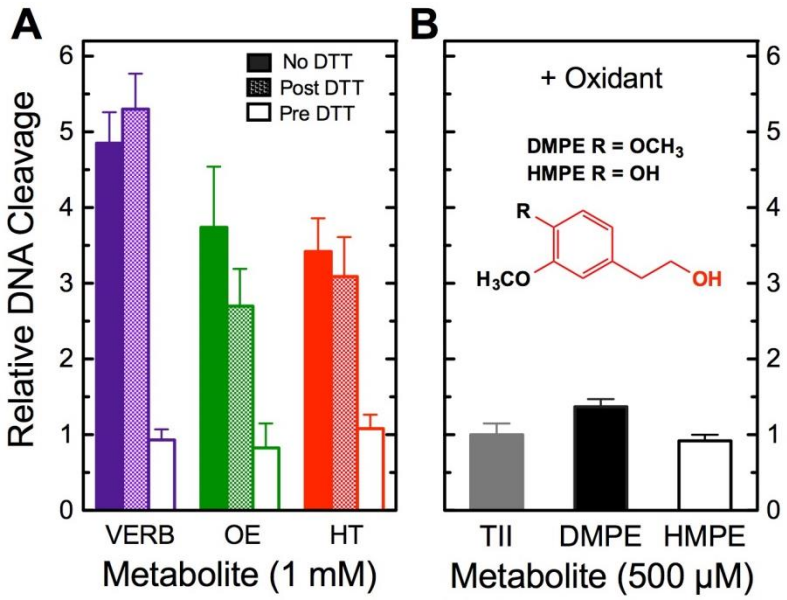


Figure 19. Ability of olive metabolites to enhance DNA cleavage as covalent topoisomerase II poisons. A) Effects of DTT on the ability of olive metabolites to enhance DNA cleavage mediated by topoisomerase IIα. DNA cleavage reactions were carried out in the absence of DTT (filled bars, No DTT), in the presence of 100 µM DTT that was added after the cleavage–ligation equilibrium was established (stippled bars, Post DTT), or in the presence of 100 µM DTT that was added at the start of the reaction (open bars, Pre DTT). Reaction mixtures contained 1 mM hydroxytyrosol (HT; red), oleuropein (OE; green), or verbascoside (VERB; purple). DNA cleavage levels were calculated relative to a control reaction that contained no metabolite. B) Effects of 3,4-dimethoxyphenylethanol and 4-hydroxy-3-methoxyphenylethanol on topoisomerase IIα-mediated DNA cleavage. The effects of 500 µM 3,4-dimethoxyphenylethanol (black bar) or 4-hydroxy-3-methoxyphenylethanol (white bar) on the cleavage of negatively supercoiled plasmid DNA by topoisomerase IIα were determined in the presence 10 µM $K_3Fe(CN)_6$. Reactions that contained no compounds are shown in gray. DNA cleavage levels were calculated relative to a control reaction that contained no compounds or oxidant. In all cases, error bars represent standard deviations for three independent experiments.

phenylethanol, respectively). The loss of the hydroxyl groups should prevent these compounds from undergoing redox cycling.[58] Even in the presence of an oxidant, neither compound increased DNA cleavage mediated by topoisomerase IIα above baseline levels (Figure 19). These findings provide further evidence that olive metabolites require redox cycling in order to enhance topoisomerase II-mediated DNA cleavage.

Fourth, although covalent poisons enhance DNA scission when added to cleavage complexes, they irreversibly inhibit topoisomerase IIα when incubated with the enzyme prior to the addition of DNA. This inhibition is a hallmark characteristic of covalent poisons and is not seen with interfacial poisons.[57,84,102,103,114] Hydroxytyrosol, oleuropein, and verbascoside (1 mM) all inhibited the DNA cleavage activity of topoisomerase IIα when added to reaction mixtures prior to the addition of DNA (Figure 20, left). Rates of enzyme inactivation were increased by the presence of $K_3Fe(CN)_6$ (right), despite the fact that the concentration of the olive metabolites (10 μM) was 100–fold lower than used in the absence of the oxidant.

Fifth, covalent topoisomerase II poisons are believed to enhance enzyme-mediated DNA cleavage, at least in part, by affecting the N-terminal protein clamp.[102,111] Consequently, they require the presence of the N-terminal domain (but not the C-terminal domain) of the protein in order to exert their effects. Interfacial topoisomerase II poisons, such as etoposide, require neither the N- nor the C-terminal protein domains, and enhance DNA cleavage even in a protein construct that contains only the catalytic core.[98] In order to determine which protein domains are required for hydroxytyrosol, oleuropein, and verbascoside to poison topoisomerase II, their effects on DNA cleavage mediated by topoisomerase IIα constructs lacking the C-terminal domain or both the N- and C-

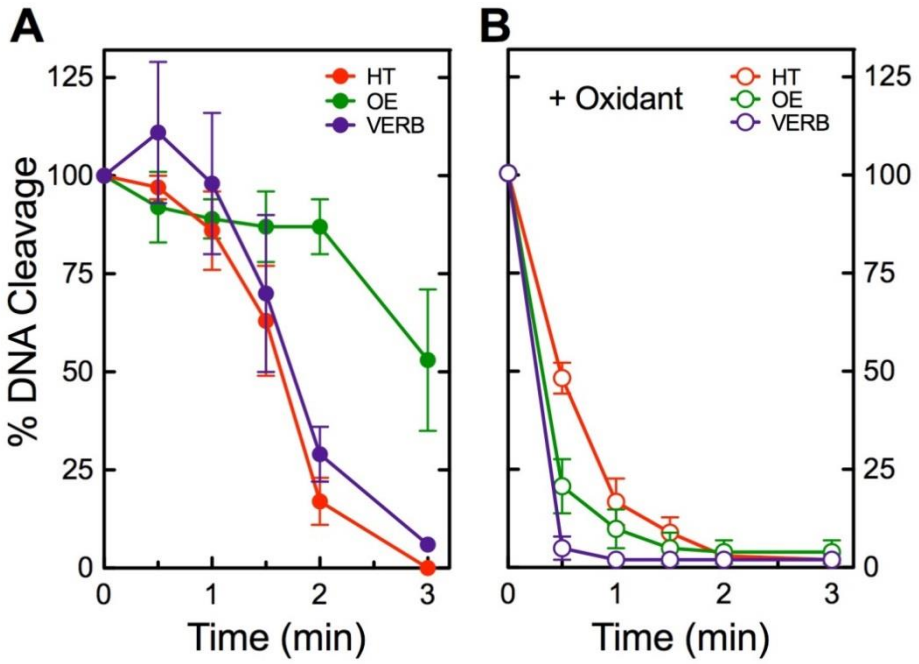


Figure 20. Ability of olive metabolites to inhibit topoisomerase IIα activity prior to DNA addition. The effects of hydroxytyrosol (HT; red), oleuropein (OE; green), and verbascoside (VERB; purple) are shown. Metabolites were incubated with the human enzyme in the absence of oxidant (1 mM metabolite, closed circles, left), or in the presence of 10 µM $K_3Fe(CN)_6$ (10 µM metabolite, open circles, right). DNA cleavage levels were calculated relative to a control reaction in which the metabolite was added after the addition of DNA to assay mixtures. Error bars represent the standard deviation of at least three independent experiments.

terminal domains (catalytic core) were assessed (Figure 21). Etoposide displayed high activity against both constructs. Although, the olive metabolites retained activity against Top2αΔ1175 (Panel A), they lost their ability to enhance DNA cleavage in absence of the N-terminal domain (catalytic core, Panel B). The presence of oxidant did not alter this latter result.

Finally, human topoisomerase IIα was treated with hydroxytyrosol and the resulting peptides generated by tryptic digestion were analyzed by MALDI mass spectrometry (Figure 22). A change in a peptide containing cysteine 104, positioned in the ATPase domain of the enzyme, was observed following treatment with hydroxytyrosol. No significant changes were observed in peptides that did not contain cysteine residues. Taken together, the above findings provide strong evidence that hydroxytyrosol, oleuropein, and verbascoside are covalent topoisomerase II poisons.

Extracts from Olive Tree Species Enhance DNA Cleavage Mediated by Topoisomerase IIα

Hydroxytyrosol, oleuropein, and verbascoside (and other polyphenols) have been reported in several members of the olive tree family.[222-225] Therefore, it is possible that other olive tree species may also produce topoisomerase II poisons.

Eleven different species of plants from the *Oleaceae* family grow in Israel. Leaf, bark, and fruit samples were harvested from these species and 36 extracts were prepared and tested for activity against human topoisomerase IIα (Figure 23). A number of extracts (at 2 mg/mL) increased enzyme-mediated DNA cleavage. Activity against topoisomerase IIα was observed across multiple species and was found in the leaf, bark, and fruit of the

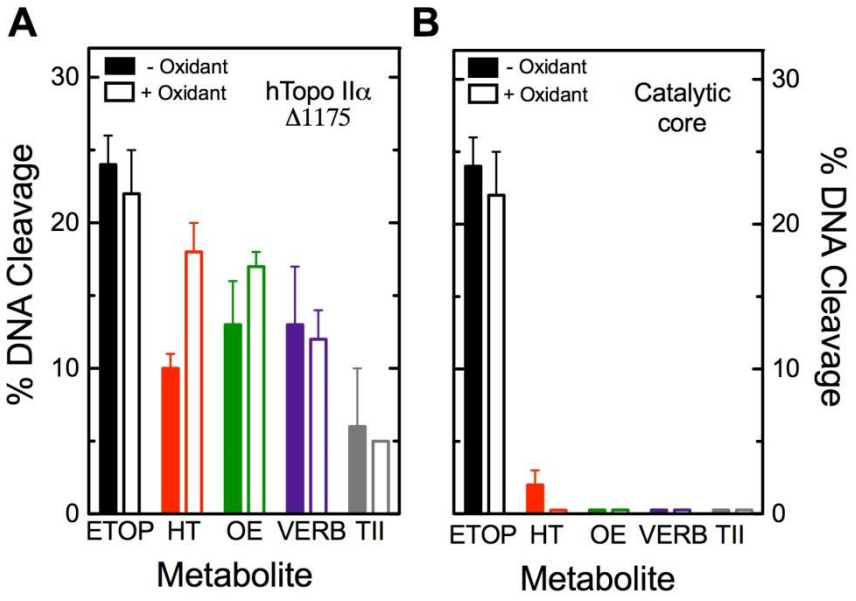


Figure 21. Requirements of topoisomerase IIα domains for olive metabolites to enhance DNA cleavage. The effects of olive metabolites on DNA cleavage mediated by topoisomerase IIα lacking the C-terminal domain (Δ1175) or both the C-terminal and N-terminal domains (Catalytic core) are shown in panels A and B, respectively. DNA cleavage reactions were carried out using 1 mM metabolites [hydroxytyrosol (HT; red), oleuropein (OE; green), or verbascoside (VERB; purple)] in the absence of oxidant (closed bars) or 10 μM metabolite in the presence of 10 μM $K_3Fe(CN)_6$. Results with no metabolites (TII, gray) or 100 μM etoposide (ETOP, black) in the absence or presence of oxidant are shown as controls. DNA cleavage levels were calculated relative to scission generated by restriction endonucease EcoRI, which was set to 100%. Error bars represent the standard deviation of at least three independent experiments. Baseline levels of DNA cleavage generated by the catalytic core are lower than those generated by full-length topoisomerase IIα.

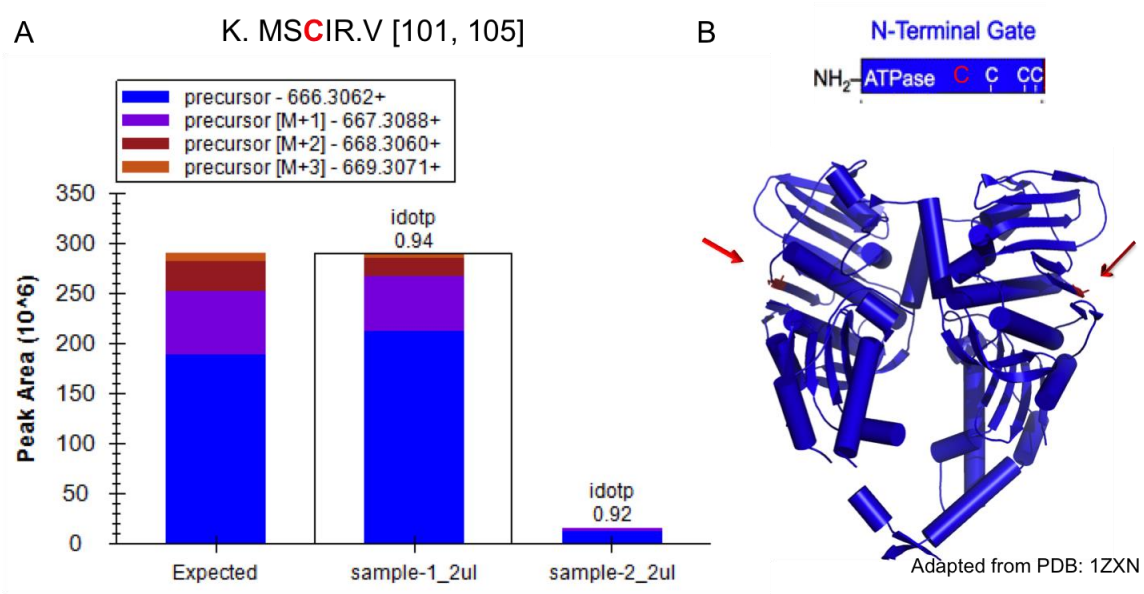


Figure 22. Mass spectrometry analysis suggesting adduction within a peptide containing cysteine residue 104. The graph on the left depicts the presence of the peptide fragment (101-105) in the expected (theoretical) versus experimental samples. Sample 1 contained only human topoisomerase IIα, while sample 2 human topoisomerase IIα treated with the covalent poison hydroxytyrosol. An Idotp >0.90 is considered statistically significant in the Skyline data processing program. (Data analyzed in part by Vanderbilt Proteomics Core) The model and schematic on the right highlights the location of cysteine residue 104 within the N-gate.

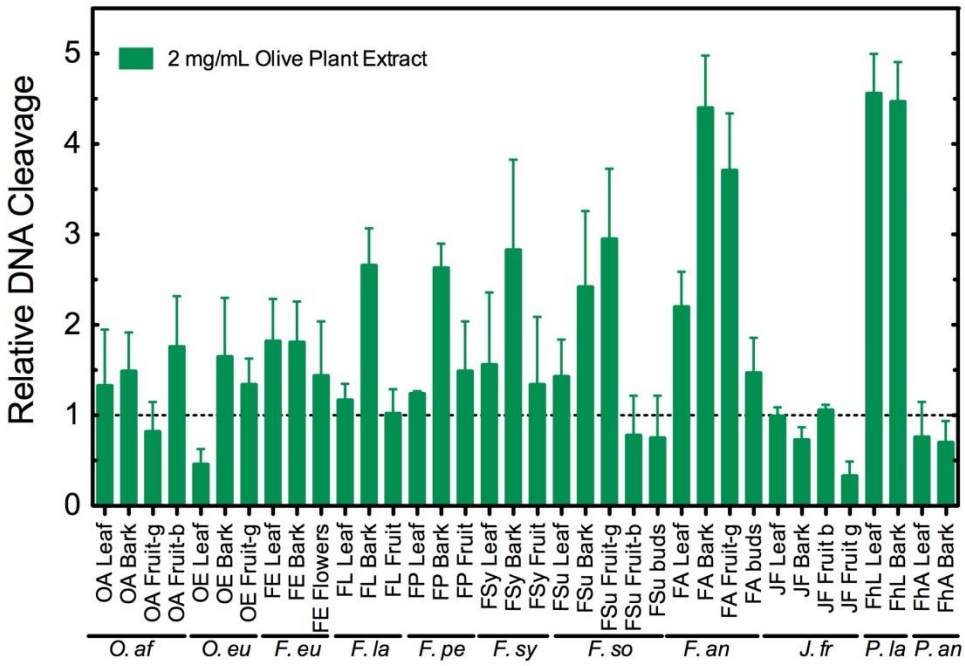


Figure 23. Effects of soluble olive tree extracts on DNA cleavage mediated by topoisomerase IIα. Thirty-six leaf, bark, and fruit extracts were prepared from 11 different species of olive trees that are indigenous to Israel. DNA cleavage reactions were carried out in the presence of 2 mg/mL extract. Abbreviations are: *Olea africana (O. af), Olea europea (O. eu), Forsithia europaea (F. eu), Fraxinus latifolia (F. la), Fraxinus pennsylvanica (F. pe), Fraxinus syriaca (F. sy), Fraxinus sogdiana (F. so), Fraxinus angustifolia (F. an), Jasminium fruiticans (J. fr), Phillyrea latifolia (P. la), Phillyrea angusifolia (P. an*). DNA cleavage levels were calculated relative to a control reaction (dashed line) that contained no extract. Error bars represent the standard deviation of at least three independent experiments.

trees. Generally, the highest levels of activity were seen in bark extracts. However, several fruit extracts also displayed high levels of DNA cleavage enhancement. These results suggest that topoisomerase II poisons are widely produced by members of the olive tree family.

To further explore the presence of topoisomerase II poisons in olive species, the ability of a commercial olive leaf extract (species not identified by the manufacturer) to enhance enzyme-mediated DNA cleavage was assessed (Figure 24). The herbal supplement enhanced DNA cleavage mediated by topoisomerase IIα nearly 5–fold at 20 mg/mL. Thus, olive metabolites can poison the type II enzyme, even in more complex formulations intended for human consumption.

Effects of Extra Virgin Olive Oils on DNA Cleavage Mediated by Topoisomerase IIα.

Hydroxytyrosol, oleuropein, and verbascoside all are present in the fruit of the olive tree and have been reported in olive oil.[222-225] Therefore, the effects of three commercial extra virgin olive oils on DNA cleavage mediated by topoisomerase IIα were assessed. Oils were pressed from a variety of subspecies of *O. europea,* including arbosana, koroneiki, or a mixture of frantoio, moraiolo, leccino, maurino, and pendolino olives. Olive oils were added to DNA cleavage assay mixtures at a final concentration of 10% by volume.

In the absence of an oxidant, no DNA cleavage enhancement was observed (data not shown). However, in the presence of 10 μM oxidant, the extra virgin olive oils increased DNA scission 2– to 4– fold (Figure 25, left). Because polyphenols are water soluble, aqueous extracts of each olive oil were tested for activity against the type II

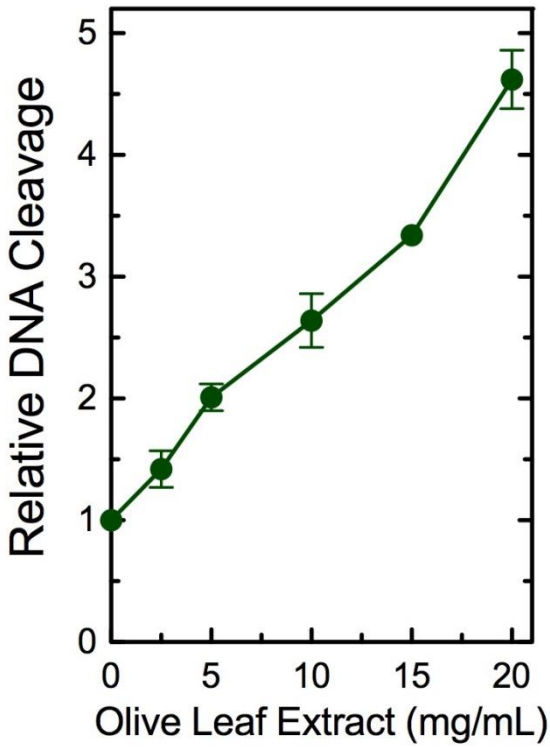


Figure 24. Effects of a commercial olive leaf extract on DNA cleavage mediated by topoisomerase II*α*. The effects of a commercial olive leaf extract (Olive leaf Plus™) on DNA cleavage mediated by the human type II enzyme are shown. DNA cleavage levels were calculated relative to a control reaction that contained no extract. Error bars represent the standard deviation of at least three independent experiments.

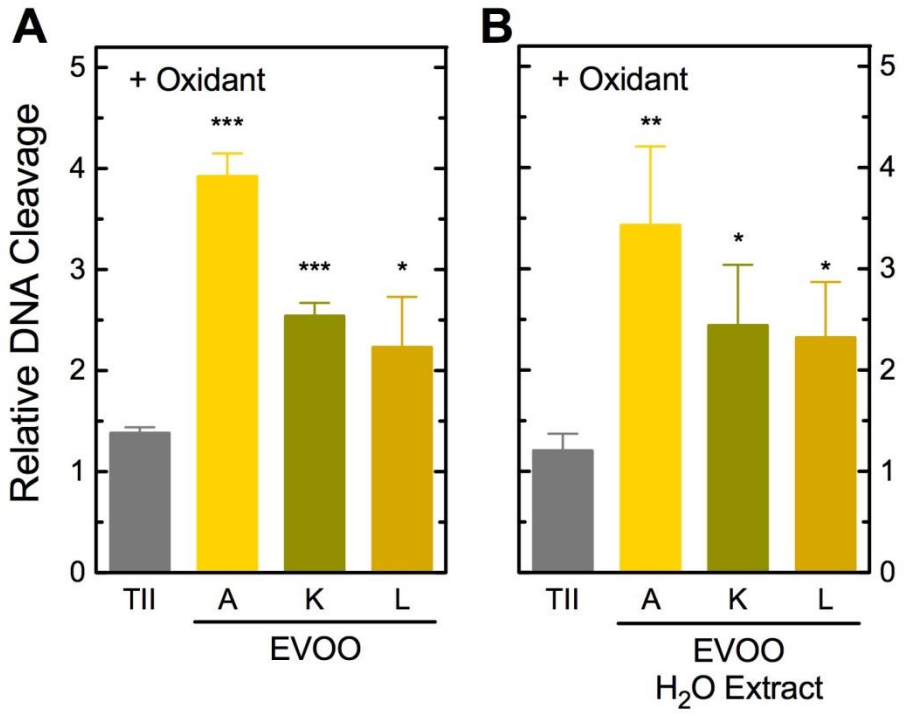


Figure 25. Extra virgin olive oils enhance DNA cleavage mediated by topoisomerase IIα. The effects of extra virgin olive oils (EVOO) or an EVOO H_2O extract on DNA cleavage mediated by the human type II enzyme are shown in panels A and B, respectively. Olive oils [Ultra Arbonsana (A, yellow), Ultra Koroneiki (K, green), or Lucini Select (L, brown)] or extracts from these oils were included in reaction mixtures at final concentrations of 10% (v/v) in the presence of 10 µM $K_3Fe(CN)_6$. Results for reactions that contained no oils or extracts, but contained oxidant (TII, gray), are shown. DNA cleavage levels were calculated relative to a control reaction that contained no olive oil or oxidant. Error bars represent the standard deviation of at least three independent experiments. Statistically significant differences are noted by asterisks (*$p < 0.05$, **$p < 0.001$, ***$p \leq 0.0001$).

enzyme (Figure 25, right). The extracts increased enzyme-mediated DNA cleavage to an extent that was similar to those of each individual extra virgin olive oil. Thus, olive products that are part of the human diet are capable of acting as topoisomerase II poisons.

Conclusions

In an effort to identify natural products that function as topoisomerase II poisons, a library of 341 extracts from Mediterranean plants was screened for the ability to enhance DNA cleavage mediated by human topoisomerase IIα. An extract from *P. latifolia* L., a member of the olive tree family, displayed high activity against the human enzyme. Further studies led to the identification of hydroxytyrosol, oleuropein, and verbascoside as covalent topoisomerase II poisons. An herbal supplement from olive leaf extracts, as well as extra virgin olive oils pressed from a variety of *O. europea* subspecies, also enhanced DNA cleavage mediated by human topoisomerase IIα. Thus, olive metabolites appear to act as topoisomerase II poisons in complex formulations intended for human dietary consumption.

Hydroxytyrosol, oleuropein, and verbascoside are well-established antioxidants. All of them induce cell cycle arrest, display anti-proliferative effects, and show activity against *in vivo* tumor models.[226-231] Furthermore, hydroxytyrosol is believed to have chemopreventative properties and currently is in clinical trials as a preventative agent for women at high risk for breast cancer.[232] Olive oil is a key component of the

Mediterranean dietary pattern, and epidemiological observations indicate that this diet has great potential for cancer prevention.[93,223,234,235]

Following consumption of 25 ml of virgin olive oil, the concentration of hydroxytyrosol in blood is estimated to be as high ~1 μM.[236] Thus, at least under oxidizing conditions, physiological levels of hydroxytyrosol are in a range at which it displays activity against human type II topoisomerases.

Although phenolic olive metabolites display complex cellular activities, it has been proposed that they exert at least some of their biological effects by redox-induced oxidation to quinones and subsequent protein adduction.[221] The finding that hydroxytyrosol, oleuropein, and verbascoside are covalent topoisomerase II poisons and that their activity is enhanced by oxidation is consistent with this hypothesis. Results of the present study suggest that the ability of these olive leaf metabolites to poison topoisomerase II may contribute to their therapeutic properties. (See appendix for follow-up that were performed to attempt to better understand the effects of covalent poisons at reactive cysteine sites)

CHAPTER IV

INHIBITION OF HUMAN DNA TOPOISOMERASE IIα BY TWO NOVEL ELLIPTICINE DERIVATIVES

Introduction

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole; $C_{17}H_{14}N_{2;}$ MW: 246.31), a natural product first isolated from the Australian evergreen tree, is an antineoplastic agent that intercalates into DNA and alters topoisomerase II activity.[100,237-244] The compound is a mild poison against topoisomerase II from *Drosophila* and *Saccharomyces cerevisiae.*[241,242] However, most studies report that ellipticine induces little, if any, DNA cleavage mediated by mammalian type II topoisomerases and inhibits enzyme activity at higher concentrations.[100,239-241] In contrast to the parent compound, several ellipticine derivatives are potent topoisomerase II poisons in mammalian systems and display anticancer activity against human breast cancer and other solid tumors.[238,244,245] Unfortunately, these compounds induce a number of adverse effects such as xerostomia, weight loss, hemolysis, and renal toxicity. Moreover, the parent compound, ellipticine, displays poor water solubility and target specificity, and drug resistance has been observed upon prolonged administration.[239,246-248]

Results and Discussion

In an effort to investigate new ellipticine-based compounds for their potential as topoisomerase II-targeted drugs, we synthesized two novel derivatives, N-methyl-5-demethyl ellipticine (ET-1) and 2-methyl-N-methyl-5-demethyl ellipticinium iodide (ET-2). Ellipticine derivatives were synthesized *via* a novel pathway shown in Fig. 26. The detailed syntheses and physical and chemical characterizations of ET-1 and ET-2 are described in the accompanying Appendix.

Briefly, ET-1 and ET-2 were generated using a nine-step synthetic pathway with a 12% overall yield (Figure 26). First, 4,9-dimethyl-9*H*-carbazole-3-carbaldehyde (1) was synthesized in five steps, according to the literature, starting from Hagemann's ester (ethyl-2-methyl-4-oxocyclohex-2-enecarboxylate).[249,250] We then generated N-methyl-5-demethyl ellipticine (ET-1; $C_{18}H_{17}N_2I$; MW: 246.3) and 2-methyl-N-methyl-5-demethyl ellipticinium iodide (ET-2; $C_{17}H_{14}N_{2;}$ MW: 388.2) in four subsequent steps.[251] Aldehyde 1 was treated with aminoacetaldehyde diethylacetal in solvent-free conditions to yield imine 2. The imine was reduced with sodium borohydride to produce amine 3, which was treated with benzene sulfonyl chloride to produce sulfonamide 4. Finally, cyclization of ET-1 was achieved by treating sulfonamide 4 with hydrochloric acid in dioxane. ET-2 was obtained by treating ET-1 with iodomethane in dimethyl formamide. Stock solutions of the test compounds (50 mM) were prepared in DMSO. ET-1 and ET-2 both were more soluble than ellipticine.

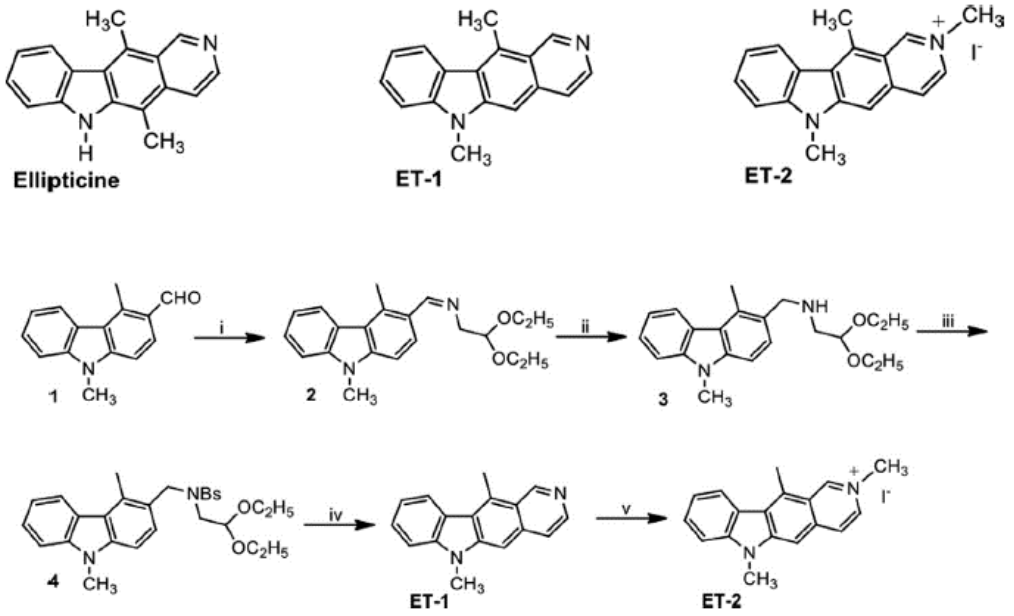


Figure 26. **Structures and synthetic pathway of the compounds ET-1 and ET-2.** Top) The structures of ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole), N-methyl-5-demethyl ellipticine (ET-1), and 2-methyl-N-methyl-5-demethyl ellipticinium iodide (ET-2) are shown. Bottom) The approach used to synthesize ellipticine derivatives ET-1 and ET-2 is shown. Reagents and Conditions: i) aminoacetaldehyde diethyl acetal, 100 °C, stirred, 4 h.; ii) $NaBH_4$, methanol, RT, stirred, 2 h.; iii) $N(C_2H_5)_3$, chloroform, benzenesulfonyl chloride, stirred, RT, 18 h.; iv) 6 N HCl, dioxane, N_2, reflux, 2 h.; v) CH_3I, DMF, stirred, RT, 5 h. (Compounds synthesized by collaborator Y. Ergun)

Effects of ET-1 and ET-2 on DNA Cleavage Mediated by Topoisomerase IIα

A number of assays were utilized to compare the effects of ET-1 and ET-2 on human topoisomerase IIα with those of the parent compound, ellipticine. First, the effects of the compounds on the overall catalytic activity of the enzyme were monitored using a decatenation assay (Figure 27). Both ET-1 and ET-2 inhibited the ability of topoisomerase IIα to unlink kinetoplast DNA (kDNA) rings and blocked enzyme activity completely at concentrations of 200-1000 μM. In contrast, ellipticine required a concentration of >5000 μM to completely inhibit the decatenation activity of the human type II enzyme.

Although topoisomerase II poisons act by enhancing levels of DNA cleavage, they still have the capacity to inhibit overall enzyme activity.[47,48] Therefore, to determine whether ET-1 and ET-2 act specifically as catalytic inhibitors or also have the capacity to act as topoisomerase II poisons, their effects on topoisomerase IIα-mediated DNA cleavage was assessed (Figure 28). As reported previously, ellipticine was a modest inhibitor of DNA cleavage (IC_{50} >200 μM).[100,240] Neither ET-1 nor ET-2 enhanced topoisomerase IIα-mediated DNA cleavage and both inhibited the DNA scission activity of the enzyme to a much greater extent than ellipticine. The IC_{50} for the two compounds were ~40 and 5 μM, respectively.

Effects of ET-1 and ET-2 on Enzyme-DNA Binding

Catalytic inhibitors can block enzyme activity at a number of different steps of the topoisomerase II catalytic cycle.[47,96 56,67,97] Compounds that inhibit the enzyme have been shown to act by blocking DNA binding, cleavage, or strand passage, or by interfering

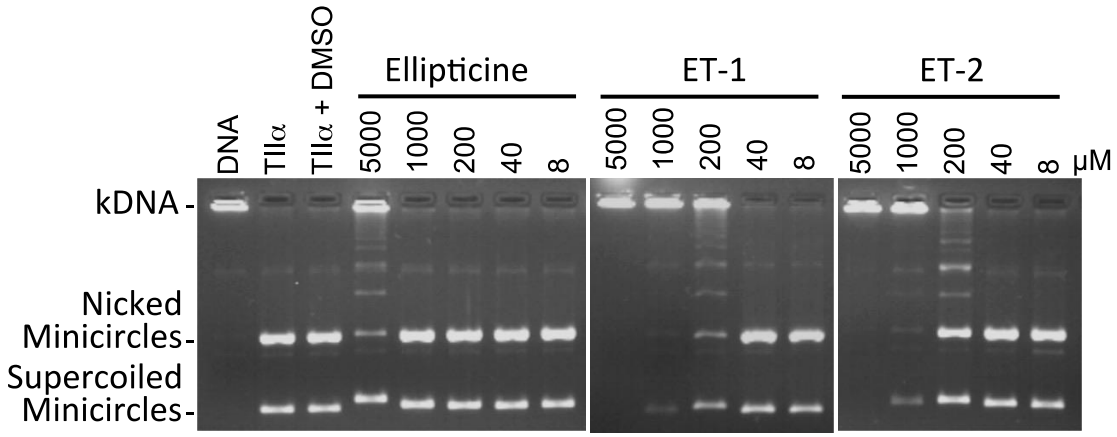


Figure 27. **Effects of ellipticine, ET-1, and ET-2 (8-5000 μM) on DNA decatenation catalyzed by human topoisomerase IIα.** Assays containing intact kDNA in the absence of topoisomerase IIα (DNA), or kDNA treated with topoisomerase IIα in the absence of ellipticine-based compounds (TIIα) or in the presence of compound diluent (TIIα + DMSO) are shown as controls. The positions of intact kDNA at the origin (kDNA), decatenated nicked kDNA minicircles, and decatenated supercoiled kDNA minicircles are indicated. Gels are representative of three independent experiments. (Decatenation assay were performed by collaborators Z. Topcu and S. Zencir)

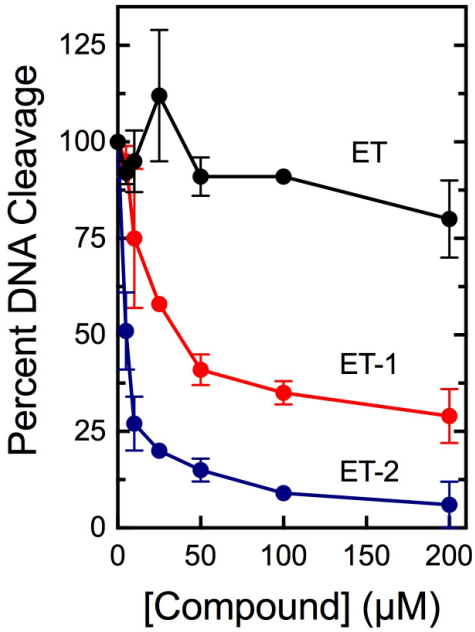


Figure 28. Effects of ellipticine, ET-1, and ET-2 on DNA cleavage mediated by human topoisomerase II*α*. Results for ellipticine (ET; black), ET-1 (ET-1; red), and ET-2 (ET-2; blue) on the generation of enzyme-mediated double-stranded DNA breaks are shown. DNA cleavage levels were calculated relative to control reactions that contained no drug and were set to 100%. Error bars represent standard deviations for at least three independent experiments.

with ATP binding/ hydrolysis. Because ET-1 and ET-2 inhibited DNA cleavage, we examined the effects of these compounds on topoisomerase IIα-DNA binding to determine whether the compounds are specific for DNA cleavage or act at a prior step in the catalytic cycle. ET-1 and ET-2 were used at 25 μM for this experiment, because DNA cleavage was inhibited substantially (~40 and 80% inhibition, respectively) at this concentration. Enzyme-DNA binding was monitored using an electrophoretic mobility shift assay (Figure 29). Neither ET-1 nor ET-2 displayed any ability to inhibit topoisomerase IIα-DNA binding at the concentration employed. In fact, ET-2 appeared to enhance enzyme-DNA binding by ~50%. Thus, we conclude that ET-1 and ET-2 act directly to inhibit DNA cleavage without altering the ability of topoisomerase IIα to bind its DNA substrate.

Effects of ET-1 and ET-2 on DNA Intercalation

The ability of ellipticine to intercalate into DNA appears to play an important role in its antineoplastic activity.[248] Because ET-1 and ET-2 are more potent inhibitors of human topoisomerase IIα than ellipticine, a topoisomerase I-DNA unwinding assay was utilized to compare the ability of these compounds to intercalate into relaxed plasmid DNA (Figure 30). As determined by the shift in the plasmid from relaxed to negatively supercoiled DNA, all of the compounds intercalated into DNA. Similar to the inhibition of DNA cleavage observed with the compounds (see Fig. 28), the relative potency of intercalation was ET-2>ET-1>>ellipticine. This finding suggests that the ability of the ellipticine derivatives to inhibit the activity of topoisomerase IIα is related to their ability to intercalate into the double helix.

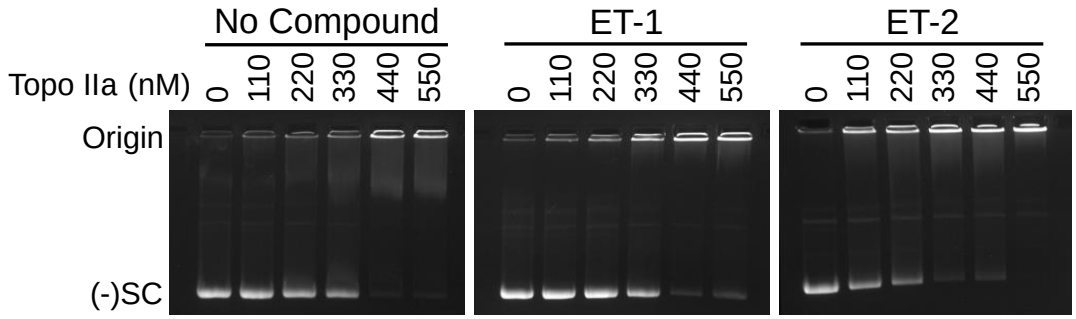


Figure 29. Effects of ET-1 and ET-2 on the binding of negatively supercoiled plasmid by human topoisomerase IIα. Results of an electrophoretic mobility shift assay are shown for an enzyme titration carried out in the presence of no compound, 25 µM ET-1, or 25 µM ET-2. Enzyme-DNA binding is indicated by the shift of the DNA from the position of negatively supercoiled [(-)SC] plasmid to the origin. Gels are representative of three independent experiments.

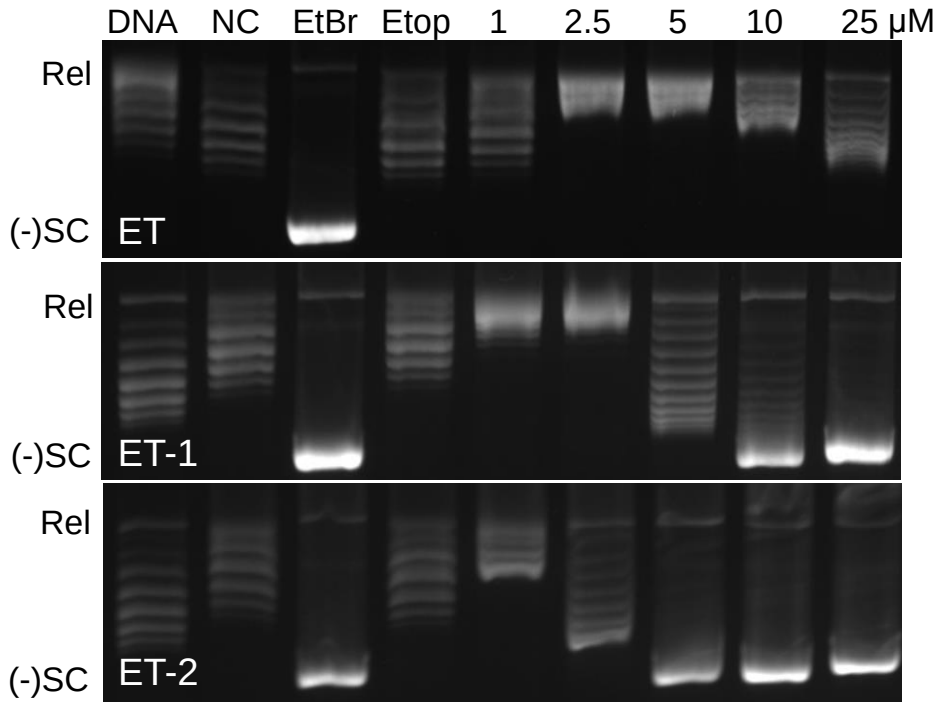


Figure 30. Intercalation of ellipticine (ET), ET-1, and ET-2 into relaxed DNA. Results of a topoisomerase I-DNA unwinding assay are shown. Intercalation is indicated by the shift in the position of the plasmid from relaxed (Rel) to negatively supercoiled [(-)SC] DNA. A strong intercalator, ethidium bromide (10 μM), and a non-intercalator, etoposide (250 μM), are shown as positive and negative controls, respectively. Assays that contained only the relaxed DNA substrate (DNA) or relaxed DNA and topoisomerase I with no compound (NC) are shown. Gels are representative of three independent experiments.

Conclusions

There is a need for the development of new anticancer drugs. Given the demonstrated antineoplastic activity of ellipticine, derivatives of this compound have the potential to display greater activity against cells or enzyme targets.[244,252,253] Results of the present study indicate that ET-1 and ET-2 are catalytic inhibitors of human topoisomerase IIα. Furthermore, both are more potent than ellipticine, and the activity of ET-1 and ET-2 toward the type II enzyme appears to be related to their enhanced ability to intercalate into DNA.

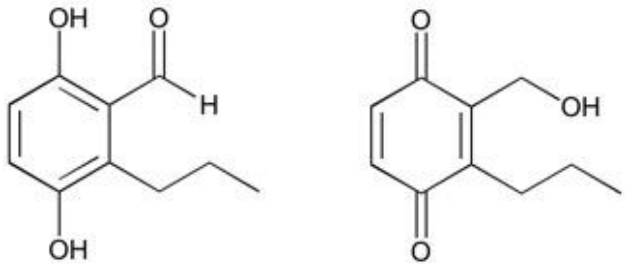
CHAPTER V

EFFECTS OF SECONDARY METABOLITES FROM THE FUNGUS *SEPTOFUSIDIUM BEROLINENSE* ON DNA CLEAVAGE MEDIATED BY HUMAN TOPOISOMERASE IIα

Introduction

Fungi are a rich source of bioactive metabolites and many of them display antineoplastic activity in cancer models.[135,254-256] Recently, two compounds that are cytotoxic to a variety of human cancer cell lines were isolated from the filamentous soil fungus, *Septofusidium berolinense*.[203] Based on mass spectroscopy and ^{13}C and ^{1}H NMR analysis, one of these metabolites was identified as 3,6-dihydroxy-2-propylbenzaldehyde (GE-1),[203] which is a hydroquinone, and the other was identified as 2-hydroxymethyl-3-propylcyclohexa-2,5-diene-1,4-dione (GE-2),[203,257] which is a quinone (Figure 31). At the present time, nothing is known about the targets that mediate the cytotoxicity of GE-1 and GE-2.

Several naturally occurring polyphenols act as topoisomerase II poisons.[57,84] These include quinones (benzoquinone, curcumin oxidation products, and thymoquinone),[58,103,108,112] hydroquinone,[108,110] and catechols (quercetin, epigallocatechin gallate, hydroxytyrosol, and oleuropein).[147,154,194] Some of these compounds work by a unique



GE-1 GE-2

Figure 31. Structures of two secondary metabolites isolated from the fungus *Septofusidium berolinense.* 3,6-dihydroxy-2-propylbenzaldehyde (GE-1) is a hydroquinone and 2-hydroxymethyl-3-propylcyclohexa-2,5-diene-1,4-dione (GE-2) is a quinone.

mechanism and are believed to enhance topoisomerase II-mediated DNA cleavage by forming covalent adducts with the enzyme. Consequently, they are referred to as *covalent topoisomerase II poisons*.[19,57]

Because GE-1 and GE-2 have structural elements consistent with some established topoisomerase II poisons, the effects of these fungal metabolites on the DNA cleavage activity of human topoisomerase IIα were assessed. Results strongly suggest that GE-2 is a covalent topoisomerase II poison. Although GE-1 does not enhance enzyme-mediated DNA scission under normal assay conditions, the presence of an oxidant converts the metabolite into an efficacious topoisomerase II poison. These findings suggest that topoisomerase IIα may be a cytotoxic target for GE-2 and potentially GE-1 in human cells.

Results and Discussion

Effects of GE-1 and GE-2 on DNA Cleavage Mediated by Topoisomerase IIα

Humans encode two isoforms of topoisomerase II, α and β.[13,18,25,27,68] Topoisomerase IIα is the isoform that is highly expressed in rapidly proliferating cells and is responsible for unlinking daughter chromosomes. The enzyme is also an important target for anticancer drugs.[13,18,19,27,56,68] Therefore, topoisomerase IIα was used to analyze the effects of GE-1 and GE-2 on enzyme-mediated DNA cleavage.

GE-2, which is a quinone, enhanced DNA cleavage mediated by topoisomerase IIα ~4–fold (Figure 32). In contrast, GE-1, which is a hydroquinone, displayed no ability to increase DNA cleavage at concentrations as high as 1 mM. Moreover, over this

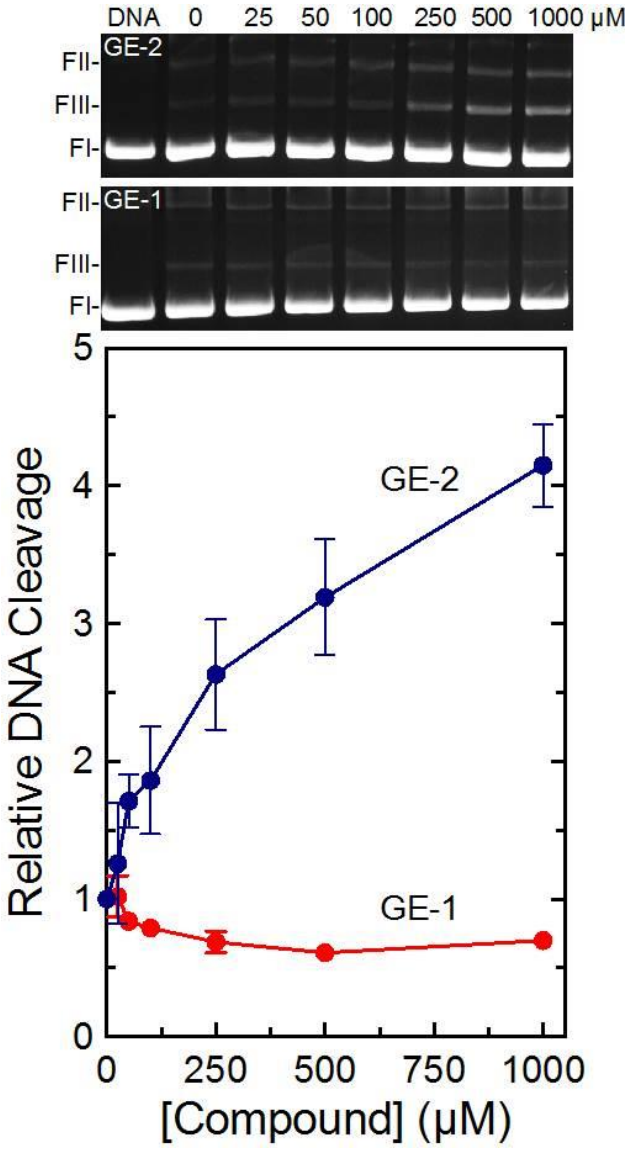


Figure 32. The effects of GE-1 and GE-2 on DNA cleavage mediated by human topoisomerase IIα. Double-stranded DNA cleavage levels in the presence of 0-1000 µM GE-1 (red) or GE-2 (blue) were calculated relative to a reaction that contained enzyme but no compound. A DNA control also is shown (DNA). Error bars represent standard deviations for at least three independent experiments. Ethidium bromide-stained agarose gels depicting DNA cleavage are shown (top). The mobility of negatively supercoiled DNA (form I; FI), nicked circular plasmid (form II; FII), and linear molecules (form III; FIII) are indicated. Gels are representative of five independent experiments.

concentration range, GE-1 inhibited DNA scission by ~40%. Previous studies found that 1,4-hydroquinone could act as a topoisomerase II poison, but required concentrations that were 5-10–fold higher than that of the corresponding 1,4-benzoquinone.[103,110] Therefore, the effects of 5 mM GE-1 on topoisomerase IIα-mediated DNA cleavage were assessed. At this concentration, GE-1 increased levels of DNA scission ~2.8–fold (data not shown). This activity of GE-1 seen at high concentrations may reflect low levels of redox cycling that converts the metabolite from a hydroquinone to a quinone under assay conditions.

The effects of GE-1 and GE-2 (0-1 mM) on DNA cleavage site utilization by topoisomerase IIα are shown in Figure 33. GE-2 increased scission at several sites and produced a banding pattern similar to that of etoposide. Consistent with the results seen with plasmid DNA, GE-1 decreased enzyme-mediated scission at all observable sites.

In order to ensure that DNA cleavage induced by GE-2 was mediated by topoisomerase IIα, three control reactions were performed in the presence of 250 μM metabolite (Figure 34). First, no DNA scission was observed when GE-2 was incubated with DNA in the absence of the type II enzyme. Second, DNA cleavage was reversed when the active site Mg^{2+} ions were chelated with EDTA prior to trapping cleavage complexes with SDS. This reversibility is not consistent with an enzyme-independent reaction. Third, cleaved plasmid products were covalently linked to topoisomerase IIα. When proteinase K treatment was omitted, the free linear DNA band disappeared and was replaced by a lower mobility smear that extended to the origin.

Finally, consistent with previous results with the quinone-based topoisomerase II poison thymoquinone,[112] GE-2 induced stable cleavage complexes with the α isoform

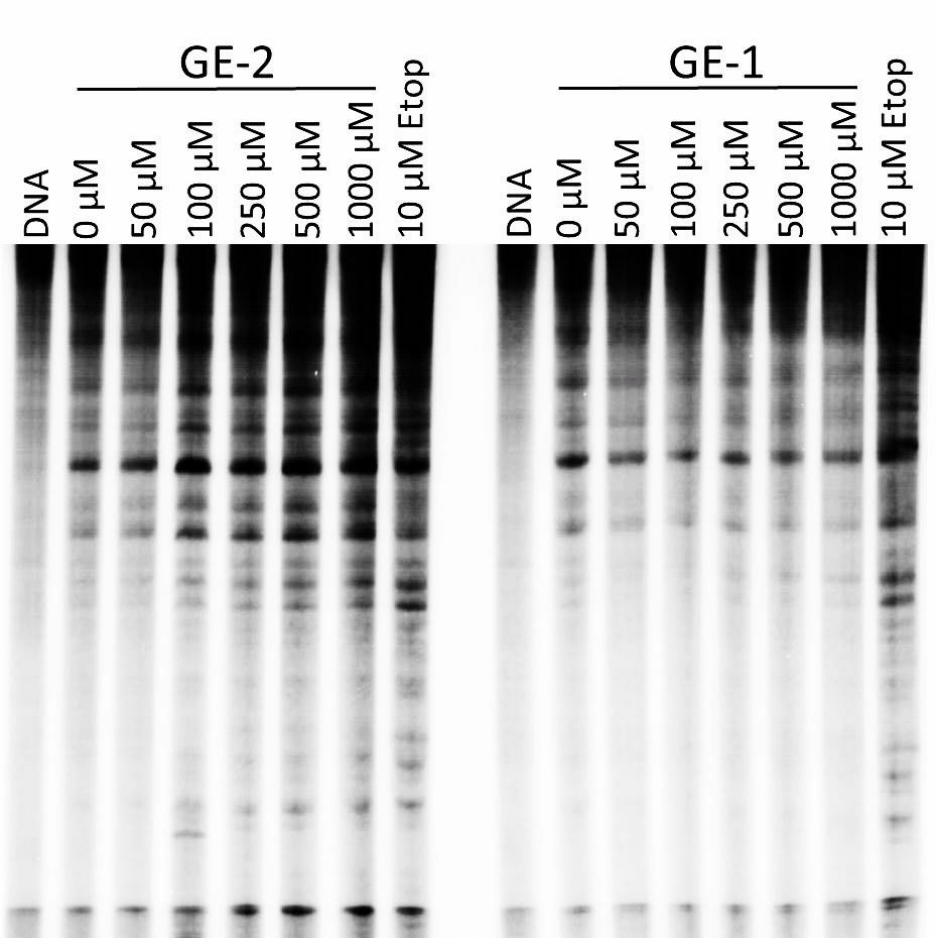


Figure 33. Effects of GE-1 and GE-2 on DNA cleavage site utilization by human topoisomerase IIα. An autoradiogram of a 6% polyacrylamide gel is shown. A singly ^{32}P-end-labeled linear 4332 bp fragment of pBR322 was used as the cleavage substrate. DNA cleavage reactions were carried out in the presence of 0-1000 µM GE-1 or GE-2, or 10 µM etoposide (as a positive control, Etop). A DNA control also is shown (DNA). Results are representative of three independent experiments.

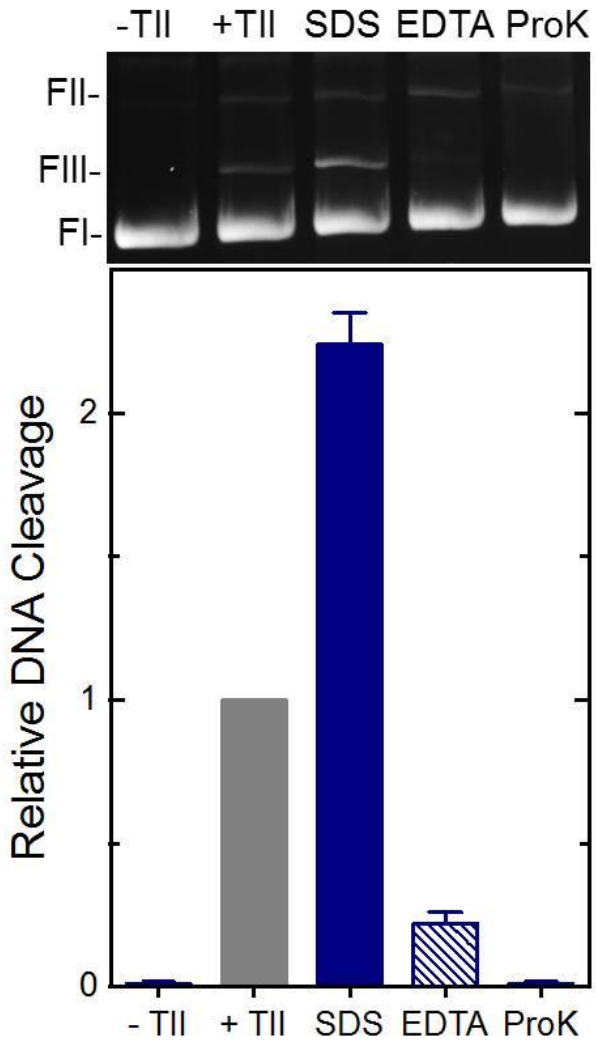


Figure 34. DNA cleavage induced by GE-2 is reversible and protein-linked. Assay mixtures contained DNA and 250 μM GE-2 in the absence of enzyme (-TII), DNA in the presence of topoisomerase IIα in the absence of GE-2 (+TII), or reaction mixtures that contained DNA, enzyme, and 250 μM GE-2 that were stopped with SDS (SDS). To determine whether the reaction was reversible, EDTA was added prior to SDS (EDTA). To determine whether the cleaved DNA was protein-linked, proteinase K treatment was omitted (ProK). The mobility of negatively supercoiled DNA (form I; FI), nicked circular plasmid (form II; FII), and linear molecules (form III; FIII) are indicated. Error bars represent the standard deviation of three independent experiments. A gel that is representative of three independent experiments is shown (top).

(not shown). The $t_{1/2}$ of DNA cleavage mediated in the presence of GE-2 was ≥ 24 h (levels of cleavage complex remaining = 58 ± 18%).

GE-2 Is a Covalent Topoisomerase II Poison

First, to assess the requirement for the N-terminal domain, the effects of 250 μM GE-1 and GE-2 on DNA cleavage mediated by the catalytic core of topoisomerase IIα were determined (Figure 35). Whereas etoposide enhanced DNA cleavage, no cleavage increase was observed in the presence of GE-1 or GE-2. In addition, neither fungal metabolite was able to diminish the effects of etoposide on DNA cleavage mediated by the catalytic core, suggesting that they were unable to compete with the interfacial poison at the active site of topoisomerase IIα (not shown).

Second, 250 μM GE-2 was incubated with topoisomerase IIα prior to the addition of DNA (Figure 36). Consistent with the actions of covalent poisons, the metabolite rapidly inactivated enzyme-mediated DNA cleavage ($t_{1/2}$ ≈ 1.5 min). In contrast, incubation with the interfacial poison etoposide (200 μM) had no effect on DNA cleavage at 3 min (Figure 36).

Third, the addition of DTT to GE-2 completely blocked its ability to enhance DNA cleavage mediated by topoisomerase IIα (Figure 37). In contrast, incubation of etoposide with DTT or other reducing agents has no significant effect on its activity against the human enzyme.[147]

Finally, the addition of oxidants to some catechols greatly enhances their potency as topoisomerase II poisons.[194] Presumably, oxidation converts the catechols to more reactive quinone-based species,[258] which can more readily adduct the type II enzyme.

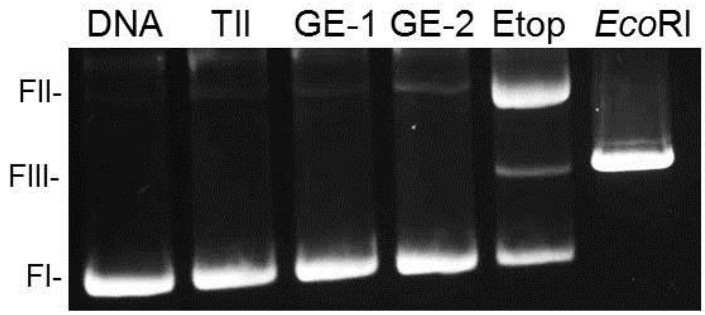


Figure 35. GE-1 and GE-2 require the N-terminal domain in order to enhance DNA cleavage mediated by topoisomerase IIα. The effects of GE-1 and GE-2 on DNA cleavage mediated by the catalytic core of human topoisomerase IIα (which lacks both the C-terminal and N-terminal domains) are shown. DNA cleavage reactions were carried out using 250 μM metabolite or 100 μM etoposide. A DNA control (DNA) and a cleavage reaction carried out in the absence of compounds (TII) also are shown. An *Eco*RI-digested plasmid control (*Eco*RI) is shown to depict the position of linear DNA and represents 100% double-stranded DNA cleavage. The gel is representative of three independent experiments.

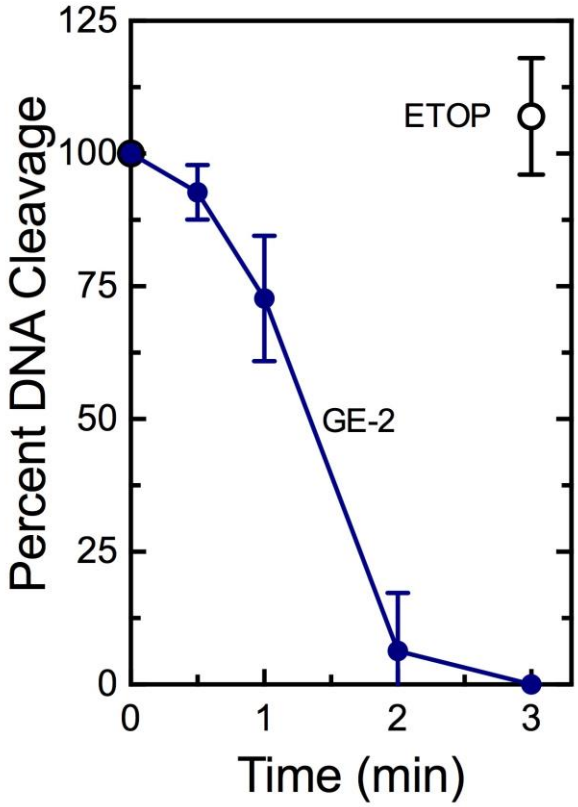


Figure 36. GE-2 inactivates human topoisomerase IIα when incubated with the enzyme prior to the addition of DNA. Cleavage reactions were initiated after topoisomerase IIα was incubated with 250 μM GE-2 for 0-3 min (blue). Double-stranded DNA cleavage levels were calculated relative to scission induced when GE-2 and the enzyme were not incubated prior to initiation of the DNA cleavage assay. Results for a control reaction in which topoisomerase IIα was incubated with 200 μM etoposide for 3 min (Etop, open black) are shown. Error bars represent standard deviations for at least three independent experiments.

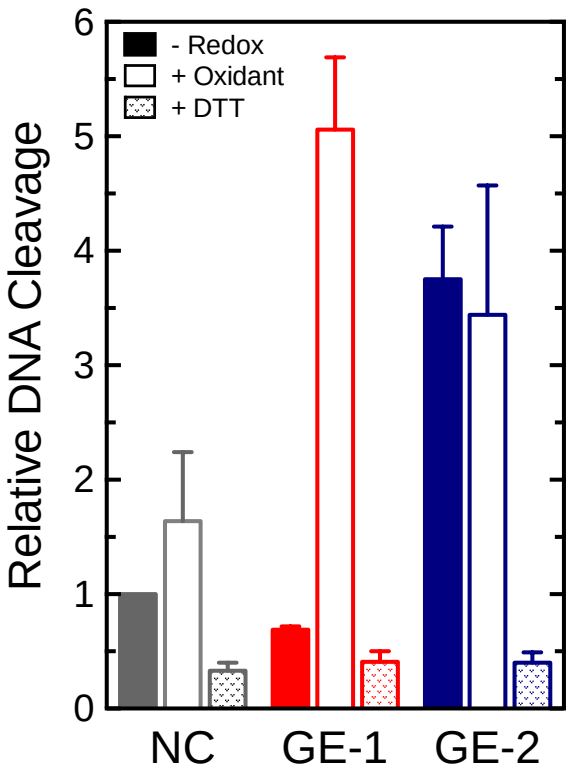


Figure 37. Effects of reducing and oxidizing reagents on the activities of GE-1 and GE-2 in topoisomerase IIα-mediated DNA cleavage reactions. DNA cleavage reactions were carried out in the absence of a reducing/oxidizing reagent (-Redox; solid bars), or in the presence of 50 μM $K_3Fe(CN)_6$ (+ oxidant; open bars) or 250 μM reducing agent (+DTT; stippled bars). Results are shown in the absence of compound [(NC); gray], or in the presence of 250 μM GE-1 (red) or GE-2 (blue). In reactions that contained the reducing or oxidizing reagent, metabolites were incubated with these reagents for 10 min prior to the start of the 6-min cleavage reaction. DNA cleavage levels were calculated relative to that of a control reaction that contained no metabolites or reducing/oxidizing reagents. Error bars represent standard deviations for at least three independent experiments.

Oxidation had no effect on the activity of GE-2, which already was a quinone (Figure 37). In marked contrast, the presence of an oxidant [$K_3Fe(CN)_6$] dramatically increased the activity of GE-1. Whereas GE-1 displayed no ability to enhance topoisomerase IIα-mediated cleavage at 1 mM (see Figure 32), oxidized GE-1 enhanced DNA scission ~5–fold at 250 μM (Figure 37).

Conclusions

Fungal metabolites are a rich source of compounds with biological activity.[135,254-256] Recently, two compounds with antineoplastic activity were isolated from the filamentous soil fungus, *S. berolinense*. GE-2, a quinone-based compound, enhanced DNA cleavage mediated by topoisomerase IIα in a concentration range at which it was reported to be cytotoxic to human cancer cell lines.[203] Furthermore, the compound displayed all of the hallmarks of a covalent topoisomerase II poison. GE-1, a hydroquinone-based compound, displayed little activity toward the human enzyme except at high concentrations. However, its activity and potency improved considerably under oxidizing conditions. Results of the present study suggest that topoisomerase IIα may play a role in mediating at least some of the antineoplastic effects of these fungal metabolites.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Nature is an attractive source for new therapeutic candidate compounds, due to the chemical scaffold diversity found in millions of species of plants, animals, marine organisms and microorganisms. In this regard, plant-based drugs have molded the basis of traditional medicine for millennia. Over the years natural products have become the single most productive source of leads for the development of drugs.[139,140] Notably, nearly half of the anticancer drugs approved internationally have been developed from either natural products or their derivatives.[135,140,141]

Natural therapies, such as the use of the plants or plant derived natural products have proven beneficial to combat cancer, even though some of the specific cellular targets are ill-defined. With an estimated 80-85% of the global population still relying on traditional medicines for their primary healthcare needs and the number of side effects associated with the use of synthetic drugs, it is worthwhile to understand current medicinal herbs and further identify potential natural products that have effects on specific cellular targets and characterize their mechanism of action.[138]

Along with traditional and modern medicine, natural dietary agents including fruits, vegetables, and spices have demonstrated the ability to suppress cancers.[93,141,146] Recent studies suggest that the consumption of food rich in fruits, vegetables and spices

have a lower incidence of cancers (stomach, esophagus, lung, oral cavity and pharynx, endometrium, pancreas and colon). Although the advancement of research has led to some progressive alternative treatments, a vast amount of information remains to be exposed, verified, and applied.

The research described in this dissertation was carried out to help uncover natural metabolites that target human topoisomerase IIα and further characterize the mechanism of topoisomerase II covalent poisons. Further identification and characterization of new topoisomerase II poisons may provide a platform for developing new anticancer and chemopreventative therapies.

Olive Metabolites as Topoisomerase II-Targeting Anticancer Agents

The Mediterranean basin is home to a biologically diverse plant biome and is a particularly rich source of botanicals with medicinal properties.[93,216,217] Phytochemicals derived from Mediterranean plant species have been shown to provide a variety of health benefits and display anti-inflammatory, anticancer, cardioprotective, and chemopreventative properties.[93,216,217]

Therefore, in an effort to discover novel phytochemicals with activity against human type II topoisomerases, research was carried out on a blind screen of a library of 341 Mediterranean plant extracts to determine whether any of them increased levels of DNA cleavage mediated by topoisomerase IIα. As described in chapter III, an extract from *Phillyrea latifolia* L., a member of the *Oleaceae* family of olive trees, displayed high activity against the human enzyme. A search of previously published metabolomics

studies on the specific plant specimen identified several polyphenols that could be potential candidates for topoisomerase II poisons. Hydroxytyrosol, oleuropein, and verbascoside were determined to act as covalent topoisomerase II poisons. Additionally, the activities of these compounds against the enzyme were accentuated under oxidizing conditions. Furthermore, water-soluble extracts of an herbal supplement from olive leaf extracts, as well as extra virgin olive oils pressed from a variety of *O. europea* subspecies, also enhanced DNA cleavage mediated by human topoisomerase IIα. Thus, olive metabolites appear to act as topoisomerase II poisons even in complex formulations intended for human dietary consumption. Results of the present study suggest that the ability of these olive metabolites to poison topoisomerase II may contribute to their therapeutic properties.

The covalent topoisomerase II poisons, hydroxytyrosol, oleuropein, and verbascoside, are well-established antioxidants and are believed to have chemopreventative properties.[226,259,260] In particular, hydroxytyrosol is the most powerful antioxidant known and is currently in clinical trials as a chemopreventative agent for women at high risk for breast cancer.[232] Researchers have found that hydroxytyrosol, oleuropein, and verbascoside induce cell cycle arrest, display anti-proliferative effects, and show activity against *in vivo* tumor models.[226-231] Furthermore, these compounds are naturally present in olives and olive oil, key components of the Mediterranean diet, and epidemiological observations have indicated that this diet has great potential for cancer prevention.[93,223,234,235]

Ellipticine Derivatives as Topoisomerase II-Targeting Anticancer Agents

The main reason for clinical interest in ellipticine and its derivatives is their effectiveness against several types of cancer, and their complete lack of hematological toxicity.[238,243,244,246] These DNA damaging compounds affect cells through multiple mechanisms, including cell cycle arrest and the initiation of apoptosis.[243,244] Ellipticine, a natural product first isolated from the Australian evergreen tree, is a well-known antineoplastic agent whose proposed mode of action is based mainly on DNA intercalation and targeting human topoisomerase II. In contrast to the parent compound, several ellipticine derivatives are potent topoisomerase II poisons in mammalian systems and display anticancer activity against human breast cancer and other solid tumors.[238,244,245] Unfortunately, these compounds induce a number of adverse effects such as dry mouth, weight loss, hemolysis, and renal toxicity. Given the demonstrated antineoplastic activity of ellipticine, derivatives of this compound have the potential to display greater activity against cells or enzyme targets.[244,252,253]

In an attempt to develop ellipticine analogs with greater anticancer activity, increased solubility, and less secondary effects, two novel C5-demethylated analogs were synthesized by our collaborators. To assess the effects of the new ellipticine-based compounds for their potential as topoisomerase II-targeted drugs, ET-1 and ET-2 were tested against human topoisomerase II activity. Results from these studies, described in chapter IV, indicate that ET-1 and ET-2 are catalytic inhibitors of human topoisomerase IIα. Furthermore, both demethylated analogs are more potent than ellipticine and the

activity of ET-1 and ET-2 toward the type II enzyme appears to be related to their enhanced ability to intercalate into DNA.

Although ET-1 and ET-2 are topoisomerase IIα inhibitors, they do not inhibit the ability of the enzyme to bind the DNA and actually appear to enhance DNA binding. Thus, these two catalytic inhibitors must be acting on another step within the topoisomerase II catalytic cycle, presumably at the DNA cleavage step.

It is clear that additional biochemical studies are needed to fully understand the role of ET-1 and ET-2. These studies might include the investigation of the effects of ET-1 and ET-2: on DNA alteration, on topoisomerase II ATPase activity, on topoisomerase II binding parameters, on cleavage site-specificity, on topoisomerase II clamp-closing activity, and on isoform specificity. Further research, such as that listed, might help to identify the specific catalytic step that is responsible for the inhibition of topoisomerase II activity and additional functions of the compounds.

Fungal Metabolites as Topoisomerase II-Targeting Anticancer Agents

Bioactive natural products can be produced by almost all types of living organisms, even soil bacteria and fungi. Soil fungi, in particular are a rich source of compounds with biological activity. These organisms have unique biochemical pathways that produce a wide range of natural products such as penicillin, cyclosporin, statins, aflatoxins, trichothecenes and ergot alkaloids.[261,262] Research has shown that filamentous fungi are frequent producers of secondary metabolites, yet only a small portion of these have been cultivated and screened for new bioactive compounds. Due to the increasing

demand for new bioactive natural products, the use of fungal diversity in the search for new bioactive molecules has become of significant interest.[254,255 263]

In a search for bioactive molecules from filamentous fungi, two compounds with antineoplastic activity were isolated from the filamentous soil fungus, *S. berolinense*. As described in chapter V, GE-2, a quinone-based compound, enhanced DNA cleavage mediated by topoisomerase IIα in a concentration range at which it is cytotoxic to human cancer cell lines.[203] Furthermore, the compound displayed all of the hallmarks of a covalent topoisomerase II poison. GE-1, a hydroquinone-based compound, displayed little activity toward the human enzyme except at high concentrations. However, its activity and potency improved considerably, under oxidizing conditions. The results presented in this study suggest that topoisomerase IIα may play a role in mediating, at least in part, the antineoplastic effects of these two fungal metabolites.

A number of fungal metabolites have been identified as healthcare agents (antibiotics, immunosuppressants, hypocholesterolemics, and antifungals). However, soil fungi have been scarcely explored and could contain potential novel anticancer molecules. Further systematic screening (phytochemical, biochemical, and bioinformatics approaches) of filamentous soil fungi species for novel metabolites and identification of their cellular targets and effects may help to identify new anticancer agents from soil fungi.

APPENDIX

POSSIBLE COVALENT ADDUCTION SITES ON HUMAN TOPOISOMERASE IIα

Introduction

Many quinone-based metabolites (1,4-benzoquinone, N-acetyl-p-benzoquinone imine (NAPQI), polychlorinated biphenyl quinones (PCB), oxidized curcumin metabolites, and (Thymoquinone) and isothiocyanates have been found to enhance topoisomerase IIα-mediated DNA cleavage through a covalent poison mechanism.[58,84,102-105,112,114,264] Quinone-based compounds can naturally be produced in the body as a result of metabolism either by reactive oxygen species or modifying enzyme. These compounds can also be a product of the species' metabolism from which it came from. Quinone-based and isothiocyanates compounds are highly reactive and have been found to act on topoisomerase II either by inhibiting the rate of religation or by blocking the N-terminal gate by crosslinking both subunits. These resulting mechanisms could be the effect of adduction to a single site or multiple sites within the enzyme.[57,67]

Research has suggested that thiol modification is the mechanism of covalent topoisomerase poisons, and that the rate of thiol modification depends on both the chemical structure of the thiol-reactive compounds and the structural microenvironment in the vicinity of the -SH group.[84,104,105,107,265] With there being 13 cysteine residues that are located throughout human topoisomerase IIα, a single or multiple of these could be

modified by covalent topoisomerase II poisons. Identification of the key cysteine residues on topoisomerase IIα that are responsible for thiol modification have been of interest to understand the covalent topoisomerase II poison mechanism.[104,105,107,265,266] These studies may also help design novel topoisomerase IIα-selective drugs.

Initial characterization studies by Hasinoff e*t. al.* showed that topoisomerase IIα contained at least five free cysteines (170, 216, 300, 392, and 405), two disulfide-bonded cysteine pairs (427–455 and 997-1008), and a possible disulfide pair that included cysteine 733.[265]More recent research by Chen *et. al.* has further exposed and categorized the reactivities of cysteines within topoisomerase IIα as the following 1) high reactivity (>10%), C104, C216, C427, and C1145; 2) medium reactivity (2∼10%), C170, C300, C392, and C405; and 3) low reactivity (<2%), C455, C733, C862, and Cys-997/Cys-1008 (Figure 38).[266]

The mechanism by which human topoisomerase IIα is targeted by quinone drugs is most characterized.[106,113,114,116,262] Bender *et al*. investigated amino acids residues in human topoisomerase II α that are modified by quinones. Cysteine residues 170, 392 and 405, located on the N-terminal domain, and 455 located in the catalytic core were able to be modified by a quinone-based compound as assessed by mass spectrometry. These findings indicated that adduction of C392 and C405 is important for the actions of quinones against the enzyme and increased levels of cleavage complexes primarily by inhibiting DNA religation.[104] Interestingly, the C445A mutant that was proposed to partner with the accessible, yet mobile, C427 in a disulfide bond was hypersensitive to benzoquinone and other covalent poisons.[265,266]

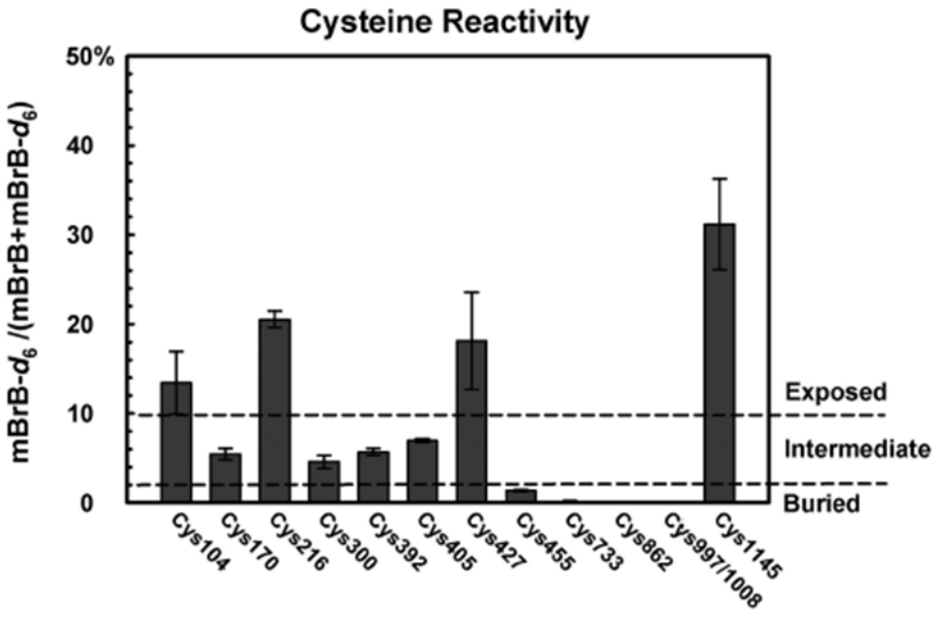


Figure 38. Reactivity of topoisomerase IIα cysteines. Previous research evaluated the cysteine accessibility within human topoisomerase IIα, using pulsed alkylation with mass spectrometric analysis. The 13 possible cysteines were categorized based on reactivity with the alkylating agent mBrB-d_6: 1) high reactivity (>10%), 2) medium reactivity (2∼10%), and 3) low reactivity. Figure reproduced from Ref. 266.

Furthermore, research in my dissertation work indicated that C104 may be adducted by hydroxytyrosol, based on differences in mass spectroscopy analysis (Figure 22). Based upon these data, the effects of cysteine to alanine mutations of residues 104, 427, and 427/455 on topoisomerase IIα activity and susceptibility to covalent poisons were investigated. The location of most of these cysteines is indicated in Figure 39.

Results and Discussion

Stability of Mutant Enzymes

The human topoisomerase IIα mutant enzymes, C427A and C427A/455A, displayed low stability through several purification techniques (affinity, affinity+p-cell, and ionic exchange+p-cell) (Figure 40). As discussed above, these two cysteine residues are suggested to pair in a disulfide bond, and C427 is a rather mobile reactive residue.[266] Thus, mutation of C427 to an alanine is the proposed cause of the enzyme instability and decreased levels of enzyme. Ultimately, this instability prevented the isolation of pure active enzyme. His-tagged wild-type, C104A and C455A human topoisomerase IIα were purified through the standard affinity+p-cell chromatography without degradation and displayed a baseline level of DNA cleavage (Figure 40).

Activity of the C104A Mutant Enzyme

Basal DNA relaxation and cleavage activity by the C104A mutant was decreased (~40%) compared to wild-type (Figures 40). Further investigation revealed that etoposide

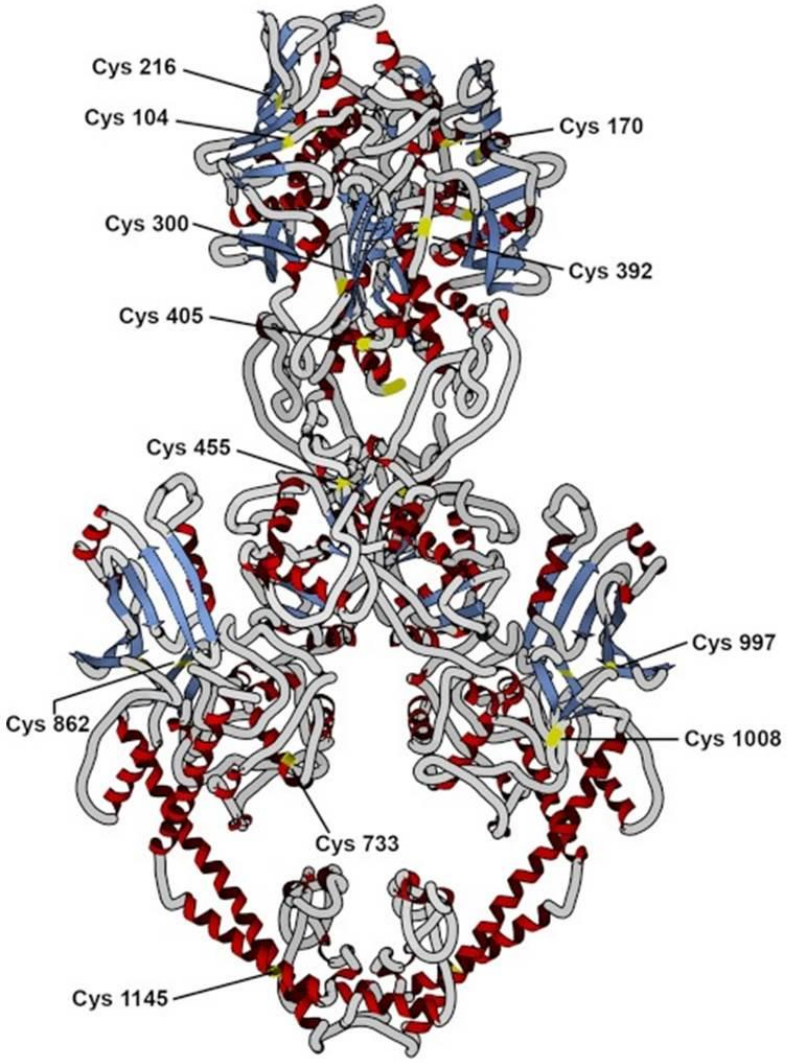


Figure 39. Location of human topoisomerase IIα cysteine residues. A homology model of human topoisomerase IIα is shown. The location of each cysteine is highlighted in yellow, except cysteine residue 427. Figure reproduced from Ref. 266.

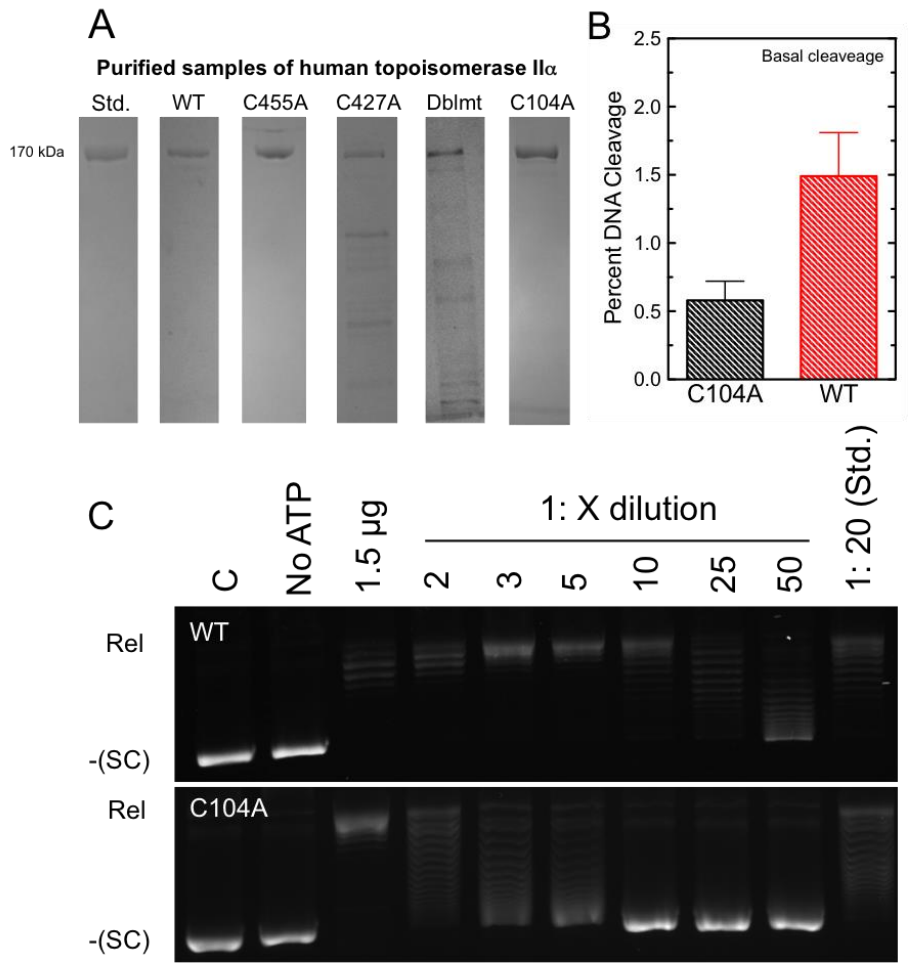


Figure 40. **Effects on protein stability and baseline cleavage activity of cysteine to alanine mutations of 104, 427, and 427/455.** A) Samples of purified wild-type and mutant human topoisomerase IIα are represented in the separate gel images (7.5% polyacrylamide). Std. represents the untagged wild-type enzyme. His-tagged wild-type and C455A were used as purification controls **B)** Baseline DNA cleavage levels of His-tagged C104A and WT enzymes were measured. Percent DNA cleavage was compared to a linear Eco RI control set to 100%. **C)** Relaxation of supercoiled pBR322 by the His-tagged C104A and WT was monitored at a range of enzyme dilutions (0-1:50). Untagged wild-type human topoisomerase IIα was used as a positive control and the enzyme at a 1:5 dilution in the absence of ATP (No ATP) was used as a negative control. All assays are representative of three or more sets of experiments.

had an effect on DNA cleavage mediated by the C104A mutant enzyme that was similar to its effects on wild-type topoisomerase IIα. (Figure 41) In contrast, the C104A mutant was hypersensitive to the covalent poison hydroxytyrosol and displayed an increased potency toward the compound as compared to the wild-type enzyme. Similarly, hydroxytyrosol induced greater levels of DNA cleavage with the C104A mutant in the presence of 10 μM oxidant than it did with the wild-type enzyme (Figure 41).

Conclusions

If we assume mutation of C104 mimics covalent adduction to this residue, two mechanisms can be proposed. 1) Modification of this residue decreases enzyme activity, but not to an extent where the residue is crucial for activity; therefore, other reactive residues must be involved. 2) Although the activity is decreased in the absence of drug, modification of this residue alters the enzyme so that it can position other reactive residues to be more accessible, leading to enzyme hypersensitivity and increased potency towards the covalent compound. In either case, C104A is not proposed to be a lone residue affected by adduction, simply a modifier of the enzyme that leads to changes in enzyme activity. However, the mechanism of action could be even more complicated than originally believed, with a mixture of reactive residues having various inhibition/activation activities.

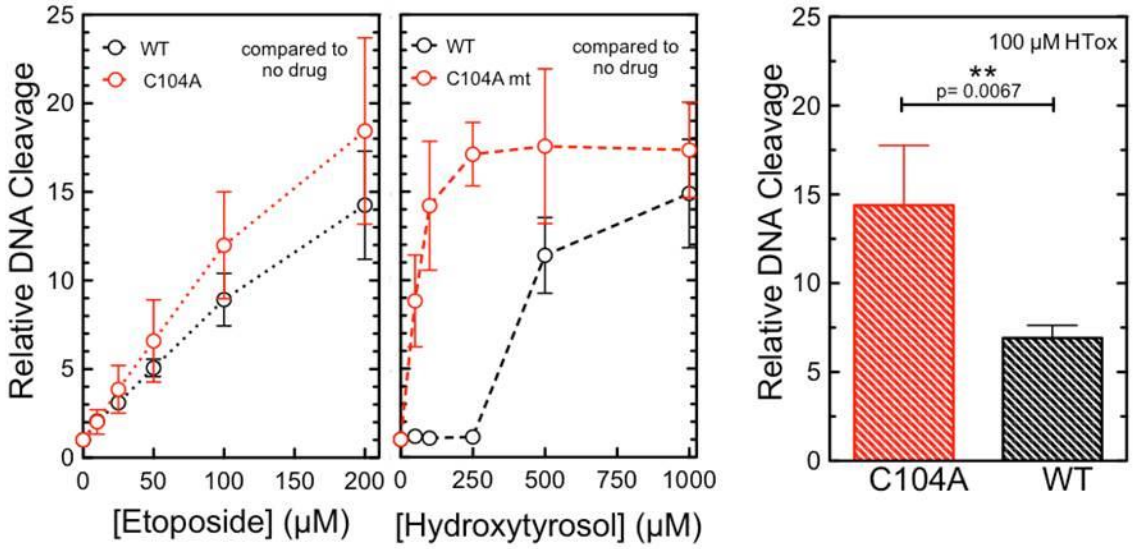


Figure 41. Effects of an interfacial and covalent poison on DNA cleavage activities mediated by His-tagged wild-type and C104A mutant human topoisomerase IIα. His-tagged C104A and WT-mediated DNA cleavage of etoposide (0-200 μM) (left), hydroxytyrosol (0-1000 μM) (middle), and 100 μM hydroxytyrosol in the presence 10 μM oxidant (right) was assessed. Relative cleavage was compared to a no drug control. All assays are representative of three or more sets of experiments.

MATERIALS AND METHODS PREPARED BY COLLOABORATORS

Materials and methods

Plant extract preparation

A library of methanol/water extracts from 341 native Mediterranean plants was used for the initial screening. Plant species were mainly from arid lands or the Tel Aviv University Botanical Garden and included plants used in traditional Bedouin medicine.[204-206] Based on results with the original library, a second library of 36 extracts from the leaf, bark, flowers, or fruit of 11 individual olive tree species was established.

Plant extracts were prepared as described by Kaiser et al.[207] Briefly, samples (1 g) of frozen plant material were ground in a pre-chilled mortar containing liquid nitrogen. Two mL of methanol/water (50/50, v/v) were added, and slurries were mixed and kept on ice for 15 min. The mixtures were centrifuged at 11,000 rpm for 5 min at room temperature using a Hermle Z160M microfuge. Supernatant liquids were stored at -80 °C for analysis. Extract concentrations were determined gravimetrically. Samples were dried *in vacuo*, de-identified, numbered, and stored at -20 °C.

Chemistry

N-((4,9-Dimethyl-9H-carbazol-3-yl)methylene)-2,2-diethoxyethanamine (2)

A mixture of carbazole aldehyde **1** (2 g, 9 mmoles) and aminoacetaldehyde diethyl acetal (1 g, 9 mmoles) was heated at 100 °C under nitrogen for 4 h. Benzene (50 mL) was added to the reaction mixture and evaporated twice in order to remove water

and volatile components. The crude product was crystallized from hexane-benzene to yield imine **2.** Yield: 3 g, 100%; mp: 95 °C; IR (KBr) ν_{max}: 2972 (CH), 1624 (C=N), 1112 (C-O-C) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.09 (t, 6H, J= 7.2 Hz, 2xCH_3), 2.94 (s, 3H, CH_3), 3.52 (q, 4H, J= 7.6 Hz, OCH_2), 3.64 (q, 4H, J= 7.6 Hz, OCH_2), 3.71 (d, 2H, J= 5.6 Hz, CH_2), 3.83 (s, 3H, NCH_3), 4.74 (t, 1H, J= 5.2 Hz, CH), 7.22 (t, 1H, J= 7.6 Hz, ArH), 7.41-7.48 (m, 2H, ArH), 7.58 (d, 1H, J= 8.4 Hz, ArH), 7.98 (d, 1H, J= 9.2 Hz, ArH), 8.20 (d, 1H, J= 8.4 Hz, ArH), 8.79 (s, 1H, =CH); Anal. Calcd for $C_{21}H_{26}N_2O_2$; C, 74.52; H, 7.74; N, 8.28. Found: C, 74.43; H, 7.67; N, 8.25.

N-((4,9-Dimethyl-9H-carbazol-3-yl)methyl)-2,2-diethoxyethanamine (3)

A solution of imine **2** (2 g, 6 mmoles) in methanol (50 mL) was cooled to 0 °C and sodium borohydride (2.3 g, 60 mmoles) was added in portions. The reaction mixture was stirred at room temperature for 2 h. The methanol was removed *in vacuo*, water was added, and the mixture was extracted with chloroform. The organic phase was dried with anhydrous magnesium sulfate and the solvent was removed *in vacuo*. The residue was recrystallized from ether to yield amine **3**. Yield: 1.85 g, 92%; mp: 117°C; IR (KBr) ν_{max}: 3220 (NH), 2935 (CH), 1150 (C-O-C) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.11 (t, 6H, J= 7.2 Hz, 2xCH_3), 2.16 (bs, 1H), 2.96 (s, 3H, CH_3), 3.51 (q, 2H, J= 7.6 Hz, OCH_2), 3.60 (q, 2H, J= 7.6 Hz, OCH_2), 3.72 (d, 2H, J= 5.6 Hz, CH_2), 3.83 (s, 3H, NCH_3), 4.35 (s, 2H, $ArCH_2$), 4.74 (t, 1H, J= 5.2 Hz, CH), 7.22 (t, 1H, J= 7.6 Hz, ArH), 7.41-7.48 (m, 2H, ArH), 7.58 (d, 1H, J= 8.4 Hz, ArH), 7.98 (d, 1H, J= 9.2 Hz, ArH), 8.20 (d, 1H, J= 8.4 Hz, ArH); Anal. Calcd for $C_{21}H_{28}N_2O_2$; C, 74.08; H, 8.29; N, 8.23. Found: C, 73.93; H, 8.24; N, 8.19.

N-(2,2-Diethoxyethyl)-N-((4,9-dimethyl-9H-carbazol-3-yl)methyl)benzenesulfonamide(4)

A solution of amine **3** (2 g, 6 mmoles) and triethyl amine (1.2 g, 12 mmoles) in chloroform (20 mL) was cooled to 0 °C. Benzenesulphonyl chloride (1.14 g, 6.5 mmoles) in chloroform (10 mL) was added drop-wise and the solution was stirred for 18 h at room temperature. The reaction mixture was diluted with chloroform and washed with hydrochloric acid (100 mL, 10%). The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed *in vacuo* and the resulting residue was isolated. The crude product was purified using silica gel and ethyl acetate-hexane (1:1). The solvent was removed *in vacuo* and the crude product was recrystallized from methanol to yield sulfonamide **4**. Yield: 2.4 g, 85%; mp: 105 °C; IR (KBr) ν_{max}: 2927 (CH), 1330 (SO), 1171 (C-O-C) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.04 (t, 6H, J= 7.2 Hz, 2xCH_3), 2.84 (s, 3H, CH_3), 3.19 (d, 2H, J= 5.6 Hz, CH_2), 3.25 (q, 2H, J= 7.6 Hz, OCH_2), 3.47 (q, 2H, J= 7.6 Hz, OCH_2), 3.79 (s, 3H, NCH_3), 4.38 (t, 1H, J= 5.6 Hz, CH), 4.70 (s, 2H, $ArCH_2$), 7.15 (d, 1H, J= 8.8 Hz, ArH), 7.23 (t, 1H, J= 8 Hz, ArH), 7.31 (d, 1H, J= 8 Hz, ArH), 7.39 (d, 1H, J= 8 Hz, ArH), 7.42-7.52 (m, 3H, ArH), 7.54 (d, 1H, J= 7.2 Hz, ArH), 7.87 (d, 2H, J= 7.2 Hz, ArH), 8.22 (d, 1H, J= 8.4 Hz, ArH); Anal. Calcd for $C_{27}H_{32}N_2SO_4$; C, 67.47; H, 6.71; N, 5.83; S, 6.67. Found: C, 67.32; H, 6.75; N, 5.81; S, 6.65.

N-Methyl 5-demethyl-ellipticine (ET-1)

Sulfonamide **4** (2 mmoles) was resuspended in dioxane (20 ml). Hydrochloric acid (2.5 mL, 6 N) was added, and the mixture was refluxed under nitrogen for 2 h. The reaction mixture was dissolved in water and extracted with chloroform. After the evaporation of solvent, the residue was chromatographed using silica gel and methanol.

The solvent was evaporated and the residue was recrystallized from ether to yield ellipticine derivative **ET-1** as a yellow powder. Yield: 400 mg, 80%; mp: 297°C; IR (KBr) ν_{max}: 3042 (CH), 2913 (CH), 1626 (C=N) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.99 (s, 3H, CH_3), 3.68 (s, 3H, NCH_3), 7.26 (t, 1H, J= 7.6 Hz, ArH), 7.45 (d, 1H, J= 8.4 Hz, ArH), 7.54 (t, 1H, J= 7.6 Hz, ArH), 7.78 (s, 1H, ArH), 8.13 (d, 2H, J= 9.2 Hz, ArH), 8.25 (d, 1H, J= 6.8 Hz, ArH), 9.66 (s, 1H, ArH); ^{13}C-NMR ($CDCl_3$, 400 MHz): 15.34, 29.65, 101.25, 109.92, 119.52, 121.28, 121.49, 123.21, 124.34, 125.47, 128.51, 128.93, 135.26, 136.21, 143.33, 143.76, 145.42; MS (m/z, %): 247 (17.75), 246 (100), 245 (44.57), 231 (10.75), 206 (7.74), 123 (22.06), 115 (11.39), 102 (12.51). Anal. Calcd for $C_{17}H_{14}N_2$; C, 82.90; H, 5.73; N, 11.37. Found: C, 82.84; H, 5.76; N, 11.39.

2-Methyl N-methyl 5-demethyl-ellipticinium iodide (ET-2)

ET-1 (500 mg, 2 mmoles) was resuspended in dimethylformamide (5 mL), and iodomethane (320 mg, 2.2 mmoles) was added. The reaction mixture was stirred at room temperature for 5 h, quenched with cold ether (5 mL), and the resulting precipitate was collected by vacuum filtration. The precipitate was washed with cold ethanol (2.5 mL) to yield **ET-2** as an orange solid. Yield: 350 mg, 44%; mp: 335°C (dec.); IR (KBr) ν_{max}: 3045 (CH), 2925 (CH), 1610 (C=N) cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.10 (s, 3H, CH_3), 3.81 (s, 3H, NCH_3), 4.37 (s, 3H, $^{+}NCH_3$), 7.35 (td, 1H, J= 7.6 and 1.2 Hz, ArH), 7.60 (d, 1H, J= 7.6 Hz, ArH), 7.65 (t, 1H, J= 7.2 Hz, ArH), 7.88 (s, 1H, ArH), 8.19 (d, 1H, J= 6.4 Hz, ArH), 8.27 (d, 2H, J= 7.6 Hz, ArH), 9.82 (s, 1H, ArH); ^{13}C-NMR (DMSO-d_6, 400 MHz): 15.49, 29.87, 47.33, 101.44, 110.19, 119.89, 121.46, 121.67, 123.72, 124.61, 125.82, 129.11, 132.87, 134.73, 135.24, 143.58, 145.65, 147.31; MS (m/z, %): 388 (100),

231 (24.75), 205 (9.62), 115 (14.48). Anal. Calcd for $C_{18}H_{17}N_2I$; C, 55.68; H, 4.41; N, 7.21. Found: C, 55.62; H, 4.43; N, 7.19

REFERENCES

1. Dahm, R. (2008) Discovering DNA: Friedrich Miescher and the early years of nucleic acid research. *Human Genetics* **122**, 565-581
2. Pray, L. (2008) Discovery of DNA structure and function: Watson and Crick. in *Nature Educ.*
3. Watson, J. D., and Crick, F. H. (1953) Molecular structure of nucleic acids; A structure for deoxyribose nucleic acid. *Nature* **171**, 737-738
4. Watson, J. D., and Crick, F. H. (1953) Genetical implications of the structure of deoxyribonucleic acid. *Nature* **171**, 964-967
5. Wang, J. C. (1971) Interaction between DNA and an Escherichia coli protein ω. *J. Mol. Biol.* **55**, 523-533
6. Boles, T. C., White, J. H., and Cozzarelli, N. R. (1990) Structure of plectonemically supercoiled DNA. *J. Mol. Biol.* **213**, 931-951
7. Bauer, W. R., Crick, F. H., and White, J. H. (1980) Supercoiled DNA. *Sci. Am.* **243**, 100-113
8. Wang, J. C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635-692
9. Espeli, O., and Marians, K. J. (2004) Untangling intracellular DNA topology. *Mol. Microbiol.* **52**, 925-931
10. Bates, A. D., and Maxwell, A. (2005) *DNA Topology*, Oxford University Press
11. Falaschi, A., Abdurashidova, G., Sandoval, O., Radulescu, S., Biamonti, G., and Riva, S. (2007) Molecular and structural transactions at human DNA replication origins. *Cell Cycle* **6**, 1705-1712

12. Azarova, A. M., Lin, R. K., Tsai, Y. C., Liu, L. F., Lin, C. P., and Lyu, Y. L. (2010) Genistein induces topoisomerase IIβ- and proteasome-mediated DNA sequence rearrangements: Implications in infant leukemia. *Biochem. Biophys. Res. Commun.* **399**, 66-71

13. Deweese, J. E., Osheroff, M. A., and Osheroff, N. (2008) DNA topology and topoisomerases: Teaching a "knotty" subject. *Biochem. Mol. Biol. Educ.* **37**, 2-10

14. Travers, A., and Muskhelishvili, G. (2007) A common topology for bacterial and eukaryotic transcription initiation? *EMBO Rep.* **8**, 147-151

15. Voet, D., and Voet, J. G. (2004) *Biochemistry*, Wiley and Sons, New York

16. Vologodskii, A. V., and Cozzarelli, N. R. (1994) Conformational and thermodynamic properties of supercoiled DNA. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 609-643

17. Mirkin, S. M. (2001) DNA Topology: Fundamentals. in *eLS*, John Wiley & Sons, Ltd. pp

18. Liu, Z., Deibler, R. W., Chan, H. S., and Zechiedrich, L. (2009) The why and how of DNA unlinking. *Nucleic Acids Res.* **37**, 661-671

19. Deweese, J. E., and Osheroff, N. (2009) The DNA cleavage reaction of topoisomerase II: Wolf in sheep's clothing. *Nucleic Acids Res.* **37**, 738-748

20. Borg, M., Mittag, T., Pawson, T., Tyers, M., Forman-Kay, J. D., and Chan, H. S. (2007) Polyelectrostatic interactions of disordered ligands suggest a physical basis for ultrasensitivity. *Proc. Natl. Acad. Sci. USA* **104**, 9650-9655

21. Mitrophanov, A. Y., and Groisman, E. A. (2008) Signal integration in bacterial two-component regulatory systems. *Genes Dev.* **22**, 2601-2611

22. Liu, L. F., and Wang, J. C. (1987) Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. USA* **84**, 7024-7027

23. Wang, J. C. (2002) Cellular roles of DNA topoisomerases: A molecular perspective. *Nat. Rev. Mol. Cell. Biol.* **3**, 430-440

24. Schvartzman, J. B., and Stasiak, A. (2004) A topological view of the replicon. *EMBO Rep.* **5**, 256-261

25. Nitiss, J. L. (2009) DNA topoisomerase II and its growing repertoire of biological functions. *Nat. Rev. Cancer* **9**, 327-337

26. Pommier, Y., Leo, E., Zhang, H., and Marchand, C. (2010) DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem. Biol.* **17**, 421-433

27. Vos, S. M., Tretter, E. M., Schmidt, B. H., and Berger, J. M. (2011) All tangled up: How cells direct, manage and exploit topoisomerase function. *Nat. Rev. Mol. Cell. Biol.* **12**, 827-841

28. Pendleton, M., Lindsey, R. H., Jr., Felix, C. A., Grimwade, D., and Osheroff, N. (2014) Topoisomerase II and leukemia. *Ann. N.Y. Acad. Sci.* **1310**, 98-110

29. Schoeffler, A. J., and Berger, J. M. (2005) Recent advances in understanding structure-function relationships in the type II topoisomerase mechanism. *Biochem. Soc. Trans.* **33**, 1465-1470

30. Nitiss, J. L. (1998) Investigating the biological functions of DNA topoisomerases in eukaryotic cells. *Biochim. Biophys. Acta.* **1400**, 63-81

31. Champoux, J. J. (2001) DNA topoisomerases: Structure, function, and mechanism. *Ann. Rev. Biochem.* **70**, 369-413

32. Leppard, J. B., and Champoux, J. J. (2005) Human DNA topoisomerase I: Relaxation, roles, and damage control. *Chromosoma* **114**, 75-85

33. Forterre, P., Gribaldo, S., Gadelle, D., and Serre, M. C. (2007) Origin and evolution of DNA topoisomerases. *Biochimie* **89**, 427-446

34. Schoeffler, A. J., and Berger, J. M. (2008) DNA topoisomerases: harnessing and constraining energy to govern chromosome topology. *Q. Rev. Biophys.* **41**, 41-101

35. Gentry, A. C. (2013) DNA topoisomerases: Type II. in *Encyc. Biol. Chem.* (Lennarz W.J., a. L. M. D. ed., Academic Press, Waltham, MA

36. Pommier, Y. (2013) Drugging topoisomerases: Lessons and challenges. *ACS Chem. Biol.* **8**, 82-95

37. Noble, C. G., and Maxwell, A. (2002) The role of GyrB in the DNA cleavage-religation reaction of DNA gyrase: a proposed two metal-ion mechanism. *J. Mol. Biol.* **318**, 361-371

38. Deweese, J. E., Burgin, A. B., and Osheroff, N. (2008) Human topoisomerase IIalpha uses a two-metal-ion mechanism for DNA cleavage. *Nucleic Acids Res.* **36**, 4883-4893

39. Stuchinskaya, T., Mitchenall, L. A., Schoeffler, A. J., Corbett, K. D., Berger, J. M., Bates, A. D., and Maxwell, A. (2009) How do type II topoisomerases use ATP hydrolysis to simplify DNA topology beyond equilibrium? Investigating the relaxation reaction of nonsupercoiling type II topoisomerases. *J. Mol. Biol.* **385**, 1397-1408

40. Sundin, O., and Varshavsky, A. (1981) Arrest of segregation leads to accumulation of highly intertwined catenated dimers: dissection of the final stages of SV40 DNA replication. *Cell* **25**, 659-669

41. Goto, T., and Wang, J. C. (1985) Cloning of yeast TOP1, the gene encoding DNA topoisomerase I, and construction of mutants defective in both DNA topoisomerase I and DNA topoisomerase II. *Proc. Natl. Acad. Sci. USA* **82**, 7178-7182

42. Goto, T., and Wang, J. C. (1984) Yeast DNA topoisomerase II is encoded by a single-copy, essential gene. *Cell* **36**, 1073-1080

43. Wyckoff, E., and Hsieh, T. S. (1988) Functional expression of a Drosophila gene in yeast: genetic complementation of DNA topoisomerase II. *Proc. Natl. Acad. Sci. USA* **85**, 6272-6276

44. Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. (1989) Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **28**, 8154-8160

45. Velez-Cruz, R., Riggins, J. N., Daniels, J. S., Cai, H., Guengerich, F. P., Marnett, L. J., and Osheroff, N. (2005) Exocyclic DNA lesions stimulate DNA cleavage mediated by human topoisomerase IIα *in vitro* and in cultured cells. *Biochemistry*

46. Berger, J. M., Fass, D., Wang, J. C., and Harrison, S. C. (1998) Structural similarities between topoisomerases that cleave one or both DNA strands. *Proc. Natl. Acad. Sci. USA* **95**, 7876-7881

47. Fortune, J. M., and Osheroff, N. (2000) Topoisomerase II as a target for anticancer drugs: When enzymes stop being nice. *Prog. Nucleic Acid Res. Mol .Biol.* **64**, 221-253

48. McClendon, A. K., and Osheroff, N. (2007) DNA topoisomerase II, genotoxicity, and cancer. *Mutat. Res.* **623**, 83-97

49. Drake, F. H., Zimmerman, J. P., McCabe, F. L., Bartus, H. F., Per, S. R., Sullivan, D. M., Ross, W. E., Mattern, M. R., Johnson, R. K., and Crooke, S. T. (1987) Purification of topoisomerase II from amsacrine-resistant P388 leukemia cells. Evidence for two forms of the enzyme. *J. Biol. Chem.* **262**, 16739-16747

50. Tsai-Pflugfelder, M., Liu, L. F., Liu, A. A., Tewey, K. M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., and Wang, J. C. (1988) Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21-22. *Proc. Natl. Acad. Sci. USA* **85**, 7177-7181

51. Chung, T. D., Drake, F. H., Tan, K. B., Per, S. R., Crooke, S. T., and Mirabelli, C. K. (1989) Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. *Proc. Natl. Acad. Sci. USA* **86**, 9431-9435

52. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ.* **2**, 209-214

53. Austin, C. A., and Marsh, K. L. (1998) Eukaryotic DNA topoisomerase IIβ. *Bioessays* **20**, 215-226

54. Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J., and Hickson, I. D. (1998) Physiological regulation of eukaryotic topoisomerase II. *Biochim. Biophys. Acta* **1400**, 121-137

55. Liu, L. F., and D'Arpa, P. (1992) Topoisomerase-targeting antitumor drugs: mechanisms of cytotoxicity and resistance. . *Important Adv. Oncol.*, 79-89

56. Nitiss, J. L. (2009) Targeting DNA topoisomerase II in cancer chemotherapy. *Nat. Rev. Cancer* **9**, 338-350

57. Ketron, A. C., and Osheroff, N. (2014) Phytochemicals as anticancer and chemopreventive topoisomerase II poisons. *Phytochem. Rev.* **13**, 19-35

58. Ketron, A. C., Gordon, O. N., Schneider, C., and Osheroff, N. (2013) Oxidative metabolites of curcumin poison human type II topoisomerases. *Biochemistry* **52**, 221-227

59. Grue, P., Grasser, A., Sehested, M., Jensen, P. B., Uhse, A., Straub, T., Ness, W., and Boege, F. (1998) Essential mitotic functions of DNA topoisomerase IIα are not adopted by topoisomerase IIβ in human H69 cells. *J. Biol. Chem.* **273**, 33660-33666

60. Corbett, K. D., and Berger, J. M. (2004) Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 95-118

61. Christensen, M. O., Larsen, M. K., Barthelmes, H. U., Hock, R., Andersen, C. L., Kjeldsen, E., Knudsen, B. R., Westergaard, O., Boege, F., and Mielke, C. (2002)

Dynamics of human DNA topoisomerases IIα and IIβ in living cells. *J. Cell Biol.* **157**, 31-44

62. Yang, X., Li, W., Prescott, E. D., Burden, S. J., and Wang, J. C. (2000) DNA topoisomerase IIβ and neural development. *Science* **287**, 131-134

63. Ju, B. G., Lunyak, V. V., Perissi, V., Garcia-Bassets, I., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2006) A topoisomerase IIβ-mediated dsDNA break required for regulated transcription. *Science* **312**, 1798-1802

64. Chen, W., Qiu, J., and Shen, Y. M. (2012) Topoisomerase IIα, rather than IIβ, is a promising target in development of anti-cancer drugs. *Drug Discov. Ther.* **6**, 230-237

65. Schmidt, B. H., Burgin, A. B., Deweese, J. E., Osheroff, N., and Berger, J. M. (2010) A novel and unified two-metal mechanism for DNA cleavage by type II and IA topoisomerases. *Nature* **465**, 641-644

66. Wendorff, T. J., Schmidt, B. H., Heslop, P., Austin, C. A., and Berger, J. M. (2012) The structure of DNA-bound human topoisomerase IIα: Conformational mechanisms for coordinating inter-subunit interactions with DNA cleavage. *J. Mol. Biol.* **424**, 109-124

67. Pogorelcnik, B., Perdih, A., and Solmajer, T. (2013) Recent developments of DNA poisons--human DNA topoisomerase IIalpha inhibitors--as anticancer agents. *Curr Pharm Des* **19**, 2474-2488

68. Chen, S. H., Chan, N.-L., and Hsieh, T.-S. (2013) New mechanistic and functional insights into DNA topoisomerases. *Ann. Rev. Biochem.* **82**, 139-170

69. Roca, J., Berger, J. M., Harrison, S. C., and Wang, J. C. (1996) DNA transport by a type II topoisomerase: direct evidence for a two-gate mechanism. *Proc. Natl. Acad. Sci. USA* **93**, 4057-4062

70. Roca, J., and Wang, J. C. (1992) The capture of a DNA double helix by an ATP-dependent protein clamp: A key step in DNA transport by type II DNA topoisomerases. *Cell* **71**, 833-840

71. Roca, J., and Wang, J. C. (1994) DNA transport by a type II DNA topoisomerase: Evidence in favor of a two-gate mechanism. *Cell* **77**, 609-616

72. Dong, K. C., and Berger, J. M. (2007) Structural basis for gate-DNA recognition and bending by type IIA topoisomerases. *Nature* **450**, 1201-1205

73. Capranico, G., and Binaschi, M. (1998) DNA sequence selectivity of topoisomerases and topoisomerase poisons. *Biochim. Biophys. Acta* **1400**, 185-194

74. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) Structure and mechanism of DNA topoisomerase II. *Nature* **379**, 225-232

75. Morais Cabral, J. H., Jackson, A. P., Smith, C. V., Shikotra, N., Maxwell, A., and Liddington, R. C. (1997) Crystal structure of the breakage-reunion domain of DNA gyrase. *Nature* **388**, 903-906

76. Laponogov, I., Pan, X. S., Veselkov, D. A., McAuley, K. E., Fisher, L. M., and Sanderson, M. R. (2010) Structural basis of gate-DNA breakage and resealing by type II topoisomerases. *PLoS One* **5**, e11338

77. Harkins, T. T., and Lindsley, J. E. (1998) Pre-steady-state analysis of ATP hydrolysis by Saccharomyces cerevisiae DNA topoisomerase II. 1. A DNA-dependent burst in ATP hydrolysis. *Biochemistry* **37**, 7292-7298

78. Wilstermann, A. M., and Osheroff, N. (2001) Positioning the 3'-DNA terminus for topoisomerase II-mediated religation. *J. Biol. Chem.* **276**, 17727-17731

79. Harkins, T. T., Lewis, T. J., and Lindsley, J. E. (1998) Pre-steady-state analysis of ATP hydrolysis by Saccharomyces cerevisiae DNA topoisomerase II. 2. Kinetic mechanism for the sequential hydrolysis of two ATP. *Biochemistry* **37**, 7299-7312

80. Zechiedrich, E. L., Christiansen, K., Andersen, A. H., Westergaard, O., and Osheroff, N. (1989) Double-stranded DNA cleavage/religation reaction of eukaryotic topoisomerase II: Evidence for a nicked DNA intermediate. *Biochemistry* **28**, 6229-6236

81. Deweese, J. E., Burgin, A. B., and Osheroff, N. (2008) Using 3'-bridging phosphorothiolates to isolate the forward DNA cleavage reaction of human topoisomerase IIalpha. *Biochemistry* **47**, 4129-4140

82. Liu, L. F. (1983) DNA topoisomerases--enzymes that catalyse the breaking and rejoining of DNA. [Review]. *Crc Critical Reviews in Biochemistry* **15**, 1-24

83. Sander, M., and Hsieh, T. (1983) Double strand DNA cleavage by type II DNA topoisomerase from Drosophila melanogaster. *J. Biol. Chem.* **258**, 8421-8428

84. Wang, H., Mao, Y., Chen, A. Y., Zhou, N., LaVoie, E. J., and Liu, L. F. (2001) Stimulation of topoisomerase II-mediated DNA damage *via* a mechanism involving protein thiolation. *Biochemistry* **40**, 3316-3323

85. Bromberg, K. D., Velez-Cruz, R., Burgin, A. B., and Osheroff, N. (2004) DNA ligation catalyzed by human topoisomerase II alpha. *Biochemistry* **43**, 13416-13423

86. Deweese, J. E., and Osheroff, N. (2009) Coordinating the two protomer active sites of human topoisomerase IIalpha: Nicks as topoisomerase II poisons. *Biochemistry* **48**, 1439-1441

87. Bender, R. P., and Osheroff, N. (2008) DNA topoisomerases as targets for the chemotherapeutic treatment of cancer. in *Checkpoint Responses in Cancer Therapy* (Dai, W. ed.), Humana Press, Totowa, New Jersey. pp 57-91

88. Wood, E. R., and Earnshaw, W. C. (1990) Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J. Cell Biol.* **111**, 2839-2850

89. Wilstermann, A. M., and Osheroff, N. (2003) Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes. *Curr. Top. Med. Chem.* **3**, 1349-1364

90. Markovits, J., Pommier, Y., Kerrigan, D., Covey, J. M., Tilchen, E. J., and Kohn, K. W. (1987) Topoisomerase II-mediated DNA breaks and cytotoxicity in relation to cell proliferation and the cell cycle in NIH 3T3 fibroblasts and L1210 leukemia cells. *Cancer Res.* **47**, 2050-2055

91. D'Arpa, P., Beardmore, C., and Liu, L. F. (1990) Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. *Cancer Res.* **50**, 6919-6924

92. Kerrigan, D., Pommier, Y., and Kohn, K. W. (1987) Protein-linked DNA strand breaks produced by etoposide and teniposide in mouse L1210 and human VA-13 and HT-29 cell lines: relationship to cytotoxicity. *NCI Monogr.*, 117-121

93. Couto, E., Boffetta, P., Lagiou, P., Ferrari, P., Buckland, G., Overvad, K., Dahm, C. C., Tjonneland, A., Olsen, A., Clavel-Chapelon, F., Boutron-Ruault, M. C., Cottet, V., Trichopoulos, D., Naska, A., Benetou, V., Kaaks, R., Rohrmann, S., Boeing, H., von Ruesten, A., Panico, S., Pala, V., Vineis, P., Palli, D., Tumino, R., May, A., Peeters, P. H., Bueno-de-Mesquita, H. B., Buchner, F. L., Lund, E., Skeie, G., Engeset, D., Gonzalez, C. A., Navarro, C., Rodriguez, L., Sanchez, M. J., Amiano, P., Barricarte, A., Hallmans, G., Johansson, I., Manjer, J., Wirfart, E., Allen, N. E., Crowe, F., Khaw, K. T., Wareham, N., Moskal, A., Slimani, N., Jenab, M., Romaguera, D., Mouw, T., Norat, T., Riboli, E., and Trichopoulou, A. (2011) Mediterranean dietary pattern and cancer risk in the EPIC cohort. *Br. J. Cancer* **104**, 1493-1499

94. Kaufmann, S. H. (1998) Cell death induced by topoisomerase-targeted drugs: more questions than answers. *Biochim. Biophys. Acta.* **1400**, 195-211

95. Felix, C. A., Kolaris, C. P., and Osheroff, N. (2006) Topoisomerase II and the etiology of chromosomal translocations. *DNA Repair (Amst.)* **5**, 1093-1108

96. Andoh, T., and Ishida, R. (1998) Catalytic inhibitors of DNA topoisomerase II. *Biochim Biophys Acta* **1400**, 155-171

97. Larsen, A. K., Escargueil, A. E., and Skladanowski, A. (2003) Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol. Ther.* **99**, 167-181

98. Lindsey, R. H., Pendleton, M., Ashley, R. E., Mercer, S. L., Deweese, J. E., and Osheroff, N. (2014) Catalytic core of human topoisomerase IIα: Insights into enzyme–DNA interactions and drug mechanism. *Biochemistry* **53**, 6595-6602

99. Pommier, Y., and Marchand, C. (2012) Interfacial inhibitors: Targeting macromolecular complexes. *Nat. Rev. Drug. Discov.* **11**, 25-36

100. Tewey, K. M., Chen, G. L., Nelson, E. M., and Liu, L. F. (1984) Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259**, 9182-9187

101. Wu, C. C., Li, T. K., Farh, L., Lin, L. Y., Lin, T. S., Yu, Y. J., Yen, T. J., Chiang, C. W., and Chan, N. L. (2011) Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. *Science* **333**, 459-462

102. Bender, R. P., Lehmler, H. J., Robertson, L. W., Ludewig, G., and Osheroff, N. (2006) Polychlorinated biphenyl quinone metabolites poison human topoisomerase IIα: Altering enzyme function by blocking the N-terminal protein gate. *Biochemistry* **45**, 10140-10152

103. Lindsey, R. H., Jr., Bromberg, K. D., Felix, C. A., and Osheroff, N. (2004) 1,4-Benzoquinone is a topoisomerase II poison. *Biochemistry* **43**, 7563-7574

104. Bender, R. P., Ham, A. J., and Osheroff, N. (2007) Quinone-induced enhancement of DNA cleavage by human topoisomerase IIα: Adduction of cysteine residues 392 and 405. *Biochemistry* **46**, 2856-2864

105. Lin, R. K., Zhou, N., Lyu, Y. L., Tsai, Y. C., Lu, C. H., Kerrigan, J., Chen, Y. T., Guan, Z., Hsieh, T. S., and Liu, L. F. (2011) Dietary isothiocyanate-induced

apoptosis *via* thiol modification of DNA topoisomerase IIα. *J. Biol. Chem.* **286**, 33591-33600

106. Frydman, B., Marton, L. J., Sun, J. S., Neder, K., Witiak, D. T., Liu, A. A., Wang, H. M., Mao, Y., Wu, H. Y., Sanders, M. M., and Liu, L. F. (1997) Induction of DNA topoisomerase II-mediated DNA cleavage by beta-lapachone and related naphthoquinones. *Cancer Res.* **57**, 620-627

107. Bender, R. P., and Osheroff, N. (2007) Mutation of cysteine residue 455 to alanine in human topoisomerase IIα confers hypersensitivity to quinones: Enhancing DNA scission by closing the N-terminal protein gate. *Chem. Res. Toxicol.* **20**, 975-981

108. Lindsey, R. H., Bender, R. P., and Osheroff, N. (2005) Stimulation of topoisomerase II-mediated DNA cleavage by benzene metabolites. *Chem. Biol. Interact.* **153-154**, 197-205

109. Baker, R. K., Kurz, E. U., Pyatt, D. W., Irons, R. D., and Kroll, D. J. (2001) Benzene metabolites antagonize etoposide-stabilized cleavable complexes of DNA topoisomerase IIalpha. *Blood* **98**, 830-833

110. Lindsey, R. H., Jr., Bender, R. P., and Osheroff, N. (2005) Effects of benzene metabolites on DNA cleavage mediated by human topoisomerase IIα: 1,4-hydroquinone is a topoisomerase II poison. *Chem. Res. Toxicol.* **18**, 761-770

111. Mondrala, S., and Eastmond, D. A. (2010) Topoisomerase II inhibition by the bioactivated benzene metabolite hydroquinone involves multiple mechanisms. *Chem. Biol. Interact.* **184**, 259-268

112. Ashley, R. E., and Osheroff, N. (2014) Natural products as topoisomerase II poisons: Effects of thymoquinone on DNA cleavage mediated by human topoisomerase IIα. *Chem. Res. Toxicol.* **27**, 787-793

113. Smith, N. A., Byl, J. A., Mercer, S. L., Deweese, J. E., and Osheroff, N. (2014) Etoposide quinone is a covalent poison of human topoisomerase IIbeta. *Biochemistry* **53**, 3229-3236

114. Bender, R. P., Lindsey, R. H., Jr., Burden, D. A., and Osheroff, N. (2004) N-acetyl-*p*-benzoquinone imine, the toxic metabolite of acetaminophen, is a topoisomerase II poison. *Biochemistry* **43**, 3731-3739

115. Jacob, D. A., Mercer, S. L., Osheroff, N., and Deweese, J. E. (2011) Etoposide quinone is a redox-dependent topoisomerase II poison. *Biochemistry* **50**, 5660-5667

116. Burden, D. A., Kingma, P. S., Froelich-Ammon, S. J., Bjornsti, M. A., Patchan, M. W., Thompson, R. B., and Osheroff, N. (1996) Topoisomerase II.Etoposide interactions direct the formation of drug-induced enzyme-DNA cleavage complexes. *J. Biol. Chem.* **271**, 29238-29244

117. Pommier, Y., and Marchand, C. (2005) Interfacial inhibitors of protein-nucleic acid interactions. *Curr. Med. Chem. Anticancer Agents* **5**, 421-429

118. Hande, K. R. (1998) Clinical applications of anticancer drugs targeted to topoisomerase II. *Biochim. Biophys. Acta.* **1400**, 173-184

119. Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., and Liu, L. F. (1984) Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J .Biol. Chem.* **259**, 13560-13566

120. Osheroff, N. (1989) Effect of antineoplastic agents on the DNA cleavage/religation reaction of eukaryotic topoisomerase II: inhibition of DNA religation by etoposide. *Biochemistry* **28**, 6157-6160

121. Hande, K. R. (1998) Etoposide: Four decades of development of a topoisomerase II inhibitor. *Eur. J. Cancer* **34**, 1514-1521

122. Willmore, E., Frank, A. J., Padget, K., Tilby, M. J., and Austin, C. A. (1998) Etoposide targets topoisomerase IIα and IIβ in leukemic cells: Isoform-specific cleavable complexes visualized and quantified *in situ* by a novel immunofluorescence technique. *Mol. Pharmacol.* **54**, 78-85

123. Mistry, A. R., Felix, C. A., Whitmarsh, R. J., Mason, A., Reiter, A., Cassinat, B., Parry, A., Walz, C., Wiemels, J. L., Segal, M. R., Ades, L., Blair, I. A., Osheroff, N., Peniket, A. J., Lafage-Pochitaloff, M., Cross, N. C., Chomienne, C., Solomon, E., Fenaux, P., and Grimwade, D. (2005) DNA topoisomerase II in therapy-related acute promyelocytic leukemia. *N. Engl. J. Med.* **352**, 1529-1538

124. Ross, W., Rowe, T., Glisson, B., Yalowich, J., and Liu, L. (1984) Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. *Cancer Res.* **44**, 5857-5860

125. Baldwin, E. L., and Osheroff, N. (2005) Etoposide, topoisomerase II and cancer. *Curr. Med. Chem. Anticancer Agents* **5**, 363-372

126. Jacob, D. A., Gibson, E. G., Mercer, S. L., and Deweese, J. E. (2013) Etoposide catechol is an oxidizable topoisomerase II poison. *Chem. Res. Toxicol.* **26**, 1156-1158

127. Wu, C.-C., Li, Y.-C., Wang, Y.-R., Li, T.-K., and Chan, N.-L. (2013) On the structural basis and design guidelines for type II topoisomerase-targeting anticancer drugs. *Nucleic Acids Res.* **41**, 10630-10640

128. Huang, N. L., and Lin, J. H. (2014) Drug-induced conformational population shifts in topoisomerase-DNA ternary complexes. *Molecules* **19**, 7415-7428

129. Drwal, M. N., Marinello, J., Manzo, S. G., Wakelin, L. P. G., Capranico, G., and Griffith, R. (2014) Novel DNA Topoisomerase IIα Inhibitors from Combined Ligand- and Structure-Based Virtual Screening. *PLoS One* **9**, e114904

130. Wilstermann, A. M., Bender, R. P., Godfrey, M., Choi, S., Anklin, C., Berkowitz, D. B., Osheroff, N., and Graves, D. E. (2007) Topoisomerase II - drug interaction domains: Identification of substituents on etoposide that interact with the enzyme. *Biochemistry* **46**, 8217-8225

131. Bender, R. P., Jablonksy, M. J., Shadid, M., Romaine, I., Dunlap, N., Anklin, C., Graves, D. E., and Osheroff, N. (2008) Substituents on etoposide that interact with human topoisomerase IIα in the binary enzyme-drug complex: Contributions to etoposide binding and activity. *Biochemistry* **47**, 4501-4509

132. Pitts, S. L., Jablonksy, M. J., Duca, M., Dauzonne, D., Monneret, C., Arimondo, P. B., Anklin, C., Graves, D. E., and Osheroff, N. (2011) Contributions of the D-Ring to the activity of etoposide against human topoisomerase IIalpha: Potential interactions with DNA in the ternary enzyme--drug-DNA complex. *Biochemistry* **50**, 5058-5066

133. Williams, D. H., Stone, M. J., Hauck, P. R., and Rahman, S. K. (1989) Why Are Secondary Metabolites (Natural Products) Biosynthesized? *J. Nat. Prod.* **52**, 1189-1208

134. Kinghorn, A. D., Chin, Y.-W., and Swanson, S. M. (2009) Discovery of Natural Product Anticancer Agents from Biodiverse Organisms. *Curr. Opin. Drug Discov. Devel.* **12**, 189-196

135. Cragg, G. M., and Newman, D. J. (2013) Natural products: A continuing source of novel drug leads. *Biochim. Biophys. Acta.* **1830**, 3670-3695

136. Prakash, O., Kumar, A., Kumar, P., and Ajeet, A. (2013) Anticancer Potential of Plants and Natural Products: A Review. *American Journal of Pharmacological Sciences* **1**, 104-115

137. Robinson, M. M., and Zhang, X. (2011) Traditional Medicines: Global situation, issues and challenges. in *The World Medicines Situation 2011* World Health Organization Press, Geneva

138. WHO. (2013) WHO Traditional Medicine Strategy 2014-2023. World Health Organization Press Geneva

139. Harvey, A. L. (2008) Natural products in drug discovery. *Drug Discov Today* **13**, 894-901

140. Newman, D. J., and Cragg, G. M. (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **75**, 311-335

141. Bhanot, A., Sharma, R., and Noolvi, M. N. (2011) Natural sources as potential anti-cancer agents: A review. *2011* **3**, 18

142. Newman, D. J., and Cragg, G. M. (2007) Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **70**, 461-477

143. Bodley, A., Liu, L. F., Israel, M., Seshadri, R., Koseki, Y., Giuliani, F. C., Kirschenbaum, S., Silber, R., and Potmesil, M. (1989) DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. *Cancer Res.* **49**, 5969-5978

144. Binaschi, M., Farinosi, R., Borgnetto, M. E., and Capranico, G. (2000) *In vivo* site specificity and human isoenzyme selectivity of two topoisomerase II-poisoning anthracyclines. *Cancer Res.* **60**, 3770-3776

145. Bandele, O. J., and Osheroff, N. (2007) Bioflavonoids as poisons of human topoisomerase IIα and IIβ. *Biochemistry* **46**, 6097-6108

146. Bandele, O. J., Clawson, S. J., and Osheroff, N. (2008) Dietary polyphenols as topoisomerase II poisons: B ring and C ring substituents determine the mechanism of enzyme-mediated DNA cleavage enhancement. *Chem. Res. Toxicol.* **21**, 1253-1260

147. Bandele, O. J., and Osheroff, N. (2008) (-)-Epigallocatechin gallate, a major constituent of green tea, poisons human type II topoisomerases. *Chem. Res. Toxicol.* **21**, 936-943

148. Kurzer, M. S., and Xu, X. (1997) Dietary phytoestrogens. *Annu Rev Nutr* **17**, 353-381

149. Scalbert, A., and Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**, 2073S-2085S

150. Galati, G., and O'Brien, P. J. (2004) Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radic. Biol. Med.* **37**, 287-303

151. Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., Datta, N., Singanusong, R., and Chen, S. S. (2004) Flavonoids in food and their health benefits. *Plant Foods Hum. Nutr.* **59**, 113-122

152. Kandaswami, C., Lee, L. T., Lee, P. P., Hwang, J. J., Ke, F. C., Huang, Y. T., and Lee, M. T. (2005) The antitumor activities of flavonoids. *In Vivo* **19**, 895-909

153. Siddiqui, I. A., Adhami, V. M., Saleem, M., and Mukhtar, H. (2006) Beneficial effects of tea and its polyphenols against prostate cancer. *Mol. Nutr. Food Res.* **50**, 130-143

154. Austin, C. A., Patel, S., Ono, K., Nakane, H., and Fisher, L. M. (1992) Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives. *Biochem. J.* **282**, 883-889

155. Bandele, O. J., and Osheroff, N. (2008) The efficacy of topoisomerase II-targeted anticancer agents reflects the persistence of drug-induced cleavage complexes in cells. *Biochemistry* **47**, 11900-11908

156. Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A., and Moon, R. (1995) Flavonoids as DNA topoisomerase antagonists and poisons: structure-activity relationships. *J. Nat. Prod.* **58**, 217-225

157. Lopez-Lazaro, M., Willmore, E., and Austin, C. A. (2010) The dietary flavonoids myricetin and fisetin act as dual inhibitors of DNA topoisomerases I and II in cells. *Mutat. Res.* **696**, 41-47

158. Markovits, J., Junqua, S., Goldwasser, F., Venuat, A. M., Luccioni, C., Beaumatin, J., Saucier, J. M., Bernheim, A., and Jacquemin-Sablon, A. (1995) Genistein resistance in human leukaemic CCRF-CEM cells: Selection of a diploid cell line with reduced DNA topoisomerase II beta isoform. *Biochem. Pharmacol.* **50**, 177-186

159. Lopez-Lazaro, M., Willmore, E., and Austin, C. A. (2007) Cells lacking DNA topoisomerase II beta are resistant to genistein. *J. Nat. Prod.* **70**, 763-767

160. Sang, S., Hou, Z., Lambert, J. D., and Yang, C. S. (2005) Redox properties of tea polyphenols and related biological activities. *Antioxid. Redox Signal.* **7**, 1704-1714

161. Isbrucker, R. A., Bausch, J., Edwards, J. A., and Wolz, E. (2006) Safety studies on epigallocatechin gallate (EGCG) preparations. Part 1: genotoxicity. *Food Chem. Toxicol.* **44**, 626-635

162. Isbrucker, R. A., Edwards, J. A., Wolz, E., Davidovich, A., and Bausch, J. (2006) Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies. *Food Chem. Toxicol.* **44**, 636-650

163. Yang, C. S., Lambert, J. D., Ju, J., Lu, G., and Sang, S. (2007) Tea and cancer prevention: molecular mechanisms and human relevance. *Toxicol. Appl. Pharmacol.* **224**, 265-273

164. Lopez-Lazaro, M., Calderon-Montano, J. M., Burgos-Moron, E., and Austin, C. A. (2011) Green tea constituents (-)-epigallocatechin-3-gallate (EGCG) and gallic acid induce topoisomerase I- and topoisomerase II-DNA complexes in cells mediated by pyrogallol-induced hydrogen peroxide. *Mutagenesis* **26**, 489-498

165. Stan, S. D., Kar, S., Stoner, G. D., and Singh, S. V. (2008) Bioactive food components and cancer risk reduction. *J. Cell. Biochem.* **104**, 339-356

166. Herr, I., and Buchler, M. W. (2010) Dietary constituents of broccoli and other cruciferous vegetables: implications for prevention and therapy of cancer. *Cancer Treat. Rev.* **36**, 377-383

167. Chung, F. L., Conaway, C. C., Rao, C. V., and Reddy, B. S. (2000) Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. *Carcinogenesis* **21**, 2287-2291

168. Warin, R., Chambers, W. H., Potter, D. M., and Singh, S. V. (2009) Prevention of mammary carcinogenesis in MMTV-neu mice by cruciferous vegetable constituent benzyl isothiocyanate. *Cancer Res.* **69**, 9473-9480

169. Singh, A. V., Xiao, D., Lew, K. L., Dhir, R., and Singh, S. V. (2004) Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts in vivo. *Carcinogenesis* **25**, 83-90

170. Griesser, M., Pistis, V., Suzuki, T., Tejera, N., Pratt, D. A., and Schneider, C. (2011) Autoxidative and cyclooxygenase-2 catalyzed transformation of the dietary chemopreventive agent curcumin. *J. Biol. Chem.* **286**, 1114-1124

171. Gordon, O. N., Luis, P. B., Ashley, R. E., Osheroff, N., and Schneider, C. (2015) Oxidative Transformation of Demethoxy- and Bisdemethoxycurcumin: Products, Mechanism of Formation, and Poisoning of Human Topoisomerase IIalpha. *Chem. Res. Toxicol.* **28**, 989-996

172. Satoskar, R. R., Shah, S. J., and Shenoy, S. G. (1986) Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with postoperative inflammation. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **24**, 651-654

173. Mahady, G. B., Pendland, S. L., Yun, G., and Lu, Z. Z. (2002) Turmeric (*Curcuma longa*) and curcumin inhibit the growth of *Helicobacter pylori*, a group 1 carcinogen. *Anticancer Res.* **22**, 4179-4181

174. Sharma, R. A., McLelland, H. R., Hill, K. A., Ireson, C. R., Euden, S. A., Manson, M. M., Pirmohamed, M., Marnett, L. J., Gescher, A. J., and Steward, W. P. (2001) Pharmacodynamic and pharmacokinetic study of oral *Curcuma* extract in patients with colorectal cancer. *Clin. Cancer Res.* **7**, 1894-1900

175. Patel, V. B., Misra, S., Patel, B. B., and Majumdar, A. P. (2010) Colorectal cancer: chemopreventive role of curcumin and resveratrol. *Nutr. Cancer* **62**, 958-967

176. Sayed, M. D. (1980) Traditional medicine in health care. *J. Ethnopharmacol.* **2**, 19-22

177. Salomi, M. J., Nair, S. C., and Panikkar, K. R. (1991) Inhibitory effects of *Nigella sativa* and saffron (*Crocus sativus*) on chemical carcinogenesis in mice. *Nutr. Cancer* **16**, 67-72

178. Burits, M., and Bucar, F. (2000) Antioxidant activity of *Nigella sativa* essential oil. *Phytother. Res.* **14**, 323-328

179. Gali-Muhtasib, H., Roessner, A., and Schneider-Stock, R. (2006) Thymoquinone: a promising anti-cancer drug from natural sources. *Int. J. Biochem. Cell Biol.* **38**, 1249-1253

180. Khader, M., Bresgen, N., and Eckl, P. M. (2009) *In vitro* toxicological properties of thymoquinone. *Food Chem. Toxicol.* **47**, 129-133

181. Joannides, M., and Grimwade, D. (2010) Molecular biology of therapy-related leukaemias. *Clin. Transl. Oncol.* **12**, 8-14

182. Joannides, M., Mays, A. N., Mistry, A. R., Hasan, S. K., Reiter, A., Wiemels, J. L., Felix, C. A., Coco, F. L., Osheroff, N., Solomon, E., and Grimwade, D. (2011) Molecular pathogenesis of secondary acute promyelocytic leukemia. *Mediterr. J. Hematol. Infect. Dis.* **3**, e2011045

183. Cowell, I. G., and Austin, C. A. (2012) Mechanism of generation of therapy related leukemia in response to anti-topoisomerase II agents. *Int. J. Environ. Res. Public Health* **9**, 2075-2091

184. Rashidi, A., and Fisher, S. I. (2013) Therapy-related acute promyelocytic leukemia: a systematic review. *Med. Oncol.* **30**, 625

185. Sung, P. A., Libura, J., and Richardson, C. (2006) Etoposide and illegitimate DNA double-strand break repair in the generation of MLL translocations: new insights and new questions. *DNA Repair (Amst.)* **5**, 1109-1118

186. Ezoe, S. (2012) Secondary leukemia associated with the anti-cancer agent, etoposide, a topoisomerase II inhibitor. *Int. J. Environ. Res. Public Health* **9**, 2444-2453

187. Ross, J. A., Davies, S. M., Potter, J. D., and Robison, L. L. (1994) Epidemiology of childhood leukemia, with a focus on infants. *Epidemiol. Rev.* **16**, 243-272

188. Ross, J. A., Potter, J. D., and Robison, L. L. (1994) Infant leukemia, topoisomerase II inhibitors, and the MLL gene. *J. Natl. Cancer Inst.* **86**, 1678-1680

189. Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., and Rowley, J. D. (2000) Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia. *Proc. Natl. Acad. Sci. USA* **97**, 4790-4795

190. Greaves, M. F. (1997) Aetiology of acute leukaemia. *Lancet* **349**, 344-349

191. Lyu, Y. L., Kerrigan, J. E., Lin, C. P., Azarova, A. M., Tsai, Y. C., Ban, Y., and Liu, L. F. (2007) Topoisomerase IIbeta mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. *Cancer Res.* **67**, 8839-8846

192. Menna, P., Salvatorelli, E., and Minotti, G. (2008) Cardiotoxicity of antitumor drugs. *Chem. Res. Toxicol.* **21**, 978-989

193. Zhang, S., Liu, X., Bawa-Khalfe, T., Lu, L. S., Lyu, Y. L., Liu, L. F., and Yeh, E. T. (2012) Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat. Med.* **18**, 1639-1642

194. Vann, K. R., Sedgeman, C. A., Gopas, J., Golan-Goldhirsh, A., and Osheroff, N. (2015) Effects of olive metabolites on DNA cleavage mediated by human type II topoisomerases. *Biochemistry* **54**, 4531-4541

195. Vann, K. R., Ergun, Y., Zencir, S., Oncuoglu, S., Osheroff, N., and Topcu, Z. (2016) Inhibition of human DNA topoisomerase IIα by two novel ellipticine derivatives. *Bioorg. Med. Chem. Lett.*

196. Vann, K. R., Ekiz, G., Zencir, S., Bedir, E., Topcu, Z., and Osheroff, N. (2016) Effects of secondary metabolites from the fungus *Septofusidium berolinense* on DNA cleavage mediated by human topoisomerase IIα. *Chem. Res. Toxicol.*

197. Worland, S. T., and Wang, J. C. (1989) Inducible overexpression, purification, and active site mapping of DNA topoisomerase II from the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**, 4412-4416

198. Wasserman, R. A., Austin, C. A., Fisher, L. M., and Wang, J. C. (1993) Use of yeast in the study of anticancer drugs targeting DNA topoisomerases: Expression of a functional recombinant human DNA topoisomerase IIα in yeast. *Cancer Res.* **53**, 3591-3596

199. Kingma, P. S., Greider, C. A., and Osheroff, N. (1997) Spontaneous DNA lesions poison human topoisomerase IIα and stimulate cleavage proximal to leukemic 11q23 chromosomal breakpoints. *Biochemistry* **36**, 5934-5939

200. Dickey, J. S., and Osheroff, N. (2005) Impact of the C-terminal domain of topoisomerase IIα on the DNA cleavage activity of the human enzyme. *Biochemistry* **44**, 11546-11154

201. Oestergaard, V. H., Bjergbaek, L., Skouboe, C., Giangiacomo, L., Knudsen, B. R., and Andersen, A. H. (2004) The transducer domain is important for clamp operation in human DNA topoisomerase IIα. *J. Biol. Chem.* **279**, 1684-1691

202. Biersack, H., Jensen, S., Gromova, I., Nielsen, I. S., Westergaard, O., and Andersen, A. H. (1996) Active heterodimers are formed from human DNA topoisomerase IIα and IIβ isoforms. *Proc. Natl. Acad. Sci. USA* **93**, 8288-8293

203. Ekiz, G., Hames, E. E., Nalbantsoy, A., and Bedir, E. (2015) Two rare quinone-type metabolites from the fungus *Septofusidium berolinense* and their biological activities. *J. Antibiot. (Tokyo)*

204. Sathiyamoorthy, P., Lugasi-Evgi, H., Van-Damme, P., Abu-Rabia, A., Gopas, J., and Golan-Goldhirsh, A. (1997) Larvicidal activity in desert plants of the Negev and Bedouin market plant products. *Pharm. Biol.* **35**, 265-273

205. Sathiyamoorthy, P., Lugasi-Evgi, H., Schlesinger, P., Kedar, I., Gopas, J., Pollack, Y., and Golan-Goldhirsh, A. (1999) Screening for cytotoxic and antimalarial activities in desert plants of the Negev and Bedouin market plant products. *Pharm. Biol.* **37**, 188-195

206. Ozer, J., Eisner, N., Ostrozhenkova, E., Bacher, A., Eisenreich, W., Benharroch, D., Golan-Goldhirsh, A., and Gopas, J. (2009) Nuphar lutea thioalkaloids inhibit the nuclear factor κB pathway, potentiate apoptosis and are synergistic with cisplatin and etoposide. *Cancer Biol. Ther.* **8**, 1860-1868

207. Kaiser, J., Yassin, M., Prakash, S., Safi, N., Agami, M., Lauw, S., Ostrozhenkova, E., Bacher, A., Rohdich, F., Eisenreich, W., Safi, J., and Golan-Goldhirsh, A. (2007) Anti-malarial drug targets: Screening for inhibitors of 2C-methyl-D-erythritol 4-phosphate synthase (IspC protein) in Mediterranean plants. *Phytomedicine* **14**, 242-249

208. Fortune, J. M., and Osheroff, N. (1998) Merbarone inhibits the catalytic activity of human topoisomerase IIα by blocking DNA cleavage. *J. Biol. Chem.* **273**, 17643-17650

209. Hawtin, R. E., Stockett, D. E., Byl, J. A., McDowell, R. S., Nguyen, T., Arkin, M. R., Conroy, A., Yang, W., Osheroff, N., and Fox, J. A. (2010) Voreloxin is an anticancer quinolone derivative that intercalates DNA and poisons topoisomerase II. *PLoS One* **5**, e10186

210. Gentry, A. C., Pitts, S. L., Jablonsky, M. J., Bailly, C., Graves, D. E., and Osheroff, N. (2011) Interactions between the etoposide derivative F14512 and human type II topoisomerases: Implications for the C4 spermine moiety in promoting enzyme-mediated DNA cleavage. *Biochemistry* **50**, 3240-3249

211. Miller, K. G., Liu, L. F., and Englund, P. T. (1981) A homogeneous type II DNA topoisomerase from HeLa cell nuclei. *J. Biol. Chem.* **256**, 9334-9339

212. Senarisoy, M., Canturk, P., Zencir, S., Baran, Y., and Topcu, Z. (2013) Gossypol interferes with both type I and type II topoisomerase activities without generating strand breaks. *Cell Biochem. Biophys.* **66**, 199-204

213. Byl, J. A., Fortune, J. M., Burden, D. A., Nitiss, J. L., Utsugi, T., Yamada, Y., and Osheroff, N. (1999) DNA topoisomerases as targets for the anticancer drug TAS-103: Primary cellular target and DNA cleavage enhancement. *Biochemistry* **38**, 15573-15579

214. Osheroff, N. (1986) Eukaryotic topoisomerase II. Characterization of enzyme turnover. *J. Biol. Chem.* **261**, 9944-9950

215. Koontz, L. (2014) Chapter One - TCA Precipitation. in *Methods Enzymol.* (Jon, L. ed.), Academic Press. pp 3-10

216. González-Tejero, M. R., Casares-Porcel, M., Sánchez-Rojas, C. P., Ramiro-Gutiérrez, J. M., Molero-Mesa, J., Pieroni, A., Giusti, M. E., Censorii, E., de

Pasquale, C., Della, A., Paraskeva-Hadijchambi, D., Hadjichambis, A., Houmani, Z., El-Demerdash, M., El-Zayat, M., Hmamouchi, M., and ElJohrig, S. (2008) Medicinal plants in the Mediterranean area: Synthesis of the results of the project Rubia. *Journal of Ethnopharmacology* **116**, 341-357

217. Zaid, H., Silbermann, M., Ben-Arye, E., and Saad, B. (2012) Greco-Arab and Islamic herbal-derived anticancer modalities: From tradition to molecular mechanisms. *Evid.-based Compl. Alt. Med.* **2012**, 349040

218. Lopez-Lazaro, M., Willmore, E., Jobson, A., Gilroy, K. L., Curtis, H., Padget, K., and Austin, C. A. (2007) Curcumin induces high levels of topoisomerase I- and II-DNA complexes in K562 leukemia cells. *J. Nat. Prod.* **70**, 1884-1888

219. Agati, G., Galardi, C., Gravano, E., Romani, A., and Tattini, M. (2002) Flavonoid distribution in tissues of *Phillyrea latifolia* L. leaves as estimated by microspectrofluorometry and multispectral fluorescence microimaging. *Photochem. Photobiol.* **76**, 350-360

220. Ayranci, E., and Erkan, N. (2013) Radical scavenging capacity of methanolic Phillyrea latifolia L. extract: Anthocyanin and phenolic acids composition of fruits. *Molecules* **18**, 1798-1810

221. Cornwell, D. G., and Ma, J. (2008) Nutritional benefit of olive oil: The biological effects of hydroxytyrosol and its arylating quinone adducts. *J Agric Food Chem* **56**, 8774-8786

222. Soler-Rivas, C., Espin, C. E., and Wichers, H. J. (2000) Review: Oleuropein and related compounds. *J. Sci. Food Agri.* **80**, 1013-1023

223. Hashim, Y. Z., Eng, M., Gill, C. I., McGlynn, H., and Rowland, I. R. (2005) Components of olive oil and chemoprevention of colorectal cancer. *Nutr. Rev.* **63**, 374-386

224. Omar, S. H. (2010) Oleuropein in olive and its pharmacological effects. *Sci. Pharm.* **78**, 133-154

225. Cicerale, S., Lucas, L., and Keast, R. (2010) Biological activities of phenolic compounds present in virgin olive oil. *Int. J. Mol. Sci.* **11**, 458-479

226. Fabiani, R., De Bartolomeo, A., Rosignoli, P., Servili, M., Montedoro, G. F., and Morozzi, G. (2002) Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *Eur J Cancer Prev* **11**, 351-358

227. Hamdi, H. K., and Castellon, R. (2005) Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. *Biochem. Biophys. Res. Commun.* **334**, 769-778

228. Zhou, L., Feng, Y., Jin, Y., Liu, X., Sui, H., Chai, N., Chen, X., Liu, N., Ji, Q., Wang, Y., and Li, Q. (2014) Verbascoside promotes apoptosis by regulating HIPK2-p53 signaling in human colorectal cancer. *BMC Cancer* **14**, 1-11

229. Lee, K. W., Kim, H. J., Lee, Y. S., Park, H. J., Choi, J. W., Ha, J., and Lee, K. T. (2007) Acteoside inhibits human promyelocytic HL-60 leukemia cell proliferation *via* inducing cell cycle arrest at G0/G1 phase and differentiation into monocyte. *Carcinogenesis* **28**, 1928-1936

230. Li, S., Han, Z., Ma, Y., Song, R., Pei, T., Zheng, T., Wang, J., Xu, D., Fang, X., Jiang, H., and Liu, L. (2014) Hydroxytyrosol inhibits cholangiocarcinoma tumor growth: An *in vivo* and *in vitro* study. *Oncol. Rep.* **31**, 145-152

231. Anter, J., Tasset, I., Demyda-Peyras, S., Ranchal, I., Moreno-Millan, M., Romero-Jimenez, M., Muntane, J., Luque de Castro, M. D., Munoz-Serrano, A., and Alonso-Moraga, A. (2014) Evaluation of potential antigenotoxic, cytotoxic and proapoptotic effects of the olive oil by-product "alperujo", hydroxytyrosol, tyrosol and verbascoside. *Mutat. Res. Genet. Toxicol. Environ. Mutagen* **772**, 25-33

232. Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Sanchez-Rovira, P., and Ramirez-Tortosa, M. C. (2010) Hydroxytyrosol: From laboratory investigations to future clinical trials. *Nutr. Rev.* **68**, 191-206

233. The Methodist Hospital System. A pilot study of hydroxytyrosol, a component of olive oil for breast cancer prevention in women at high risk of breast cancer. 2014- [cited 2015 Jan 20]. Available from:https://clinicaltrials.gov. NLM Identifier: NCT02068092.

234. Cardeno, A., Sanchez-Hidalgo, M., Cortes-Delgado, A., and Alarcon de la Lastra, C. (2013) Mechanisms involved in the antiproliferative and proapoptotic effects of unsaponifiable fraction of extra virgin olive oil on HT-29 cancer cells. *Nutrition and Cancer* **65**, 908-918

235. Escrich, E., Solanas, M., and Moral, R. (2014) Olive oil and other dietary lipids in breast cancer. *Cancer Treatment and Research* **159**, 289-309

236. Miro-Casas, E., Covas, M. I., Fito, M., Farre-Albadalejo, M., Marrugat, J., and de la Torre, R. (2003) Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. *Eur. J. Clin. Nutr.* **57**, 186-190

237. Goodwin, S., Smith, A. F., and Horning, E. C. (1959) Alkaloids of *Ochrosia elliptica* Labill. 1. *J. Am. Chem. Soc.* **81**, 1903-1908

238. Auclair, C. (1987) Multimodal action of antitumor agents on DNA: The ellipticine series. *Arch. Biochem. Biophys.* **259**, 1-14

239. Monnot, M., Mauffret, O., Simon, V., Lescot, E., Psaume, B., Saucier, J. M., Charra, M., Belehradek, J., Jr., and Fermandjian, S. (1991) DNA-drug recognition and effects on topoisomerase II-mediated cytotoxicity. A three-mode binding model for ellipticine derivatives. *J. Biol. Chem.* **266**, 1820-1829

240. Fosse, P., Rene, B., Charra, M., Paoletti, C., and Saucier, J. M. (1992) Stimulation of topoisomerase II-mediated DNA cleavage by ellipticine derivatives: Structure-activity relationship. *Mol. Pharmacol.* **42**, 590-595

241. Froelich-Ammon, S. J., Patchan, M. W., Osheroff, N., and Thompson, R. B. (1995) Topoisomerase II binds to ellipticine in the absence or presence of DNA: Characterization of enzyme-drug interactions by fluorescence spectroscopy. *J. Biol. Chem.* **270**, 14998-15004

242. Cline, S. D., Macdonald, T. L., and Osheroff, N. (1997) Azatoxin is a mechanistic hybrid of the topoisomerase II-targeted anticancer drugs etoposide and ellipticine. *Biochemistry* **36**, 13095-13101

243. Garbett, N. C., and Graves, D. E. (2004) Extending nature's leads: The anticancer agent ellipticine. *Curr. Med. Chem. Anticancer Agents* **4**, 149-172

244. Kizek, R., Adam, V., Hrabeta, J., Eckschlager, T., Smutny, S., Burda, J. V., Frei, E., and Stiborova, M. (2012) Anthracyclines and ellipticines as DNA-damaging anticancer drugs: Recent advances. *Pharmacol. Ther.* **133**, 26-39

245. Multon, E., Riou, J.-F., LeFevre, D., Ahomadegbe, J.-C., and Riou, G. (1989) Topoisomerase II-mediated DNA cleavage activity induced by ellipticines on the human tumor cell line N417. *Biochem. Pharm.* **38**, 2077-2086

246. Rouesse, J., Spielmann, M., Turpin, F., Le Chevalier, T., Azab, M., and Mondesir, J. M. (1993) Phase II study of elliptinium acetate salvage treatment of advanced breast cancer. *Eur. J. Cancer* **29A**, 856-859

247. Kattan, J., Durand, M., Droz, J. P., Mahjoubi, M., Marino, J. P., and Azab, M. (1994) Phase I study of retelliptine dihydrochloride (SR 95325 B) using a single two-hour intravenous infusion schedule. *Am. J. Clin. Oncol.* **17**, 242-245

248. Paoletti, C., Le Pecq, J. B., Dat-Xuong, N., Juret, P., Garnier, H., Amiel, J. L., and Rouesse, J. (1980) Antitumor activity, pharmacology, and toxicity of ellipticines, ellipticinium, and 9-hydroxy derivatives: Preliminary clinical trials of 2-methyl-9-hydroxy ellipticinium (NSC 264-137). *Recent Results Cancer Res.* **74**, 107-123

249. Karmakar, S., Dixit, R., Nath, A., Kumar, S., and Karmakar, S. (2012) Dilated cardiomyopathy following trastuzumab chemotherapy. *Indian J. Pharm.* **44**, 131-133

250. Ergün, Y., Patir, S., and Okay, G. (1998) Total synthesis of 5-demethylellipticine. *J. Heterocyc. Chem.* **35**, 1445-1447

251. Yokoyama, Y., Okuyama, N., Iwadate, S., Momoi, T., and Murakami, Y. (1990) Synthetic studies of indoles and related compounds. Part 22. The Vilsmeier-

Haack reaction of N-benzyl-1,2,3,4-tetrahydrocarbazoles and its synthetic application to olivacine and ellipticine. *J. Chem. Soc., Perkin Trans. 1*, 1319-1329

252. Stiborova, M., and Frei, E. (2014) Ellipticines as DNA-targeted chemotherapeutics. *Curr. Med. Chem.* **21**, 575-591

253. Russell, E. G., O'Sullivan, E. C., Miller, C. M., Stanicka, J., McCarthy, F. O., and Cotter, T. G. (2014) Ellipticine derivative induces potent cytostatic effect in acute myeloid leukaemia cells. *Invest. New Drugs* **32**, 1113-1122

254. Greve, H., Mohamed, I., Pontius, A., Kehraus, S., Gross, H., and König, G. (2010) Fungal metabolites: Structural diversity as incentive for anticancer drug development. *Phytochem. Rev.* **9**, 537-545

255. Kharwar, R. N., Mishra, A., Gond, S. K., Stierle, A., and Stierle, D. (2011) Anticancer compounds derived from fungal endophytes: Their importance and future challenges. *Nat. Prod. Rep.* **28**, 1208-1228

256. Evidente, A., Kornienko, A., Cimmino, A., Andolfi, A., Lefranc, F., Mathieu, V., and Kiss, R. (2014) Fungal metabolites with anticancer activity. *Nat. Prod. Rep.* **31**, 617-627

257. Ymele-Leki, P., Cao, S., Sharp, J., Lambert, K. G., McAdam, A. J., Husson, R. N., Tamayo, G., Clardy, J., and Watnick, P. I. (2012) A high-throughput screen identifies a new natural product with broad-spectrum antibacterial activity. *PLoS One* **7**, e31307

258. Vogna, D., Pezzella, A., Panzella, L., Napolitano, A., and d'Ischia, M. (2003) Oxidative chemistry of hydroxytyrosol: Isolation and characterisation of novel methanooxocinobenzodioxinone derivatives. *Tetrahedron Lett.* **44**, 8289-8292

259. Giner, E., Recio, M. C., Rios, J. L., Cerda-Nicolas, J. M., and Giner, R. M. (2016) Chemopreventive effect of oleuropein in colitis-associated colorectal cancer in c57bl/6 mice. *Mol Nutr Food Res* **60**, 242-255

260. Kostyuk, V. A., Potapovich, A. I., Lulli, D., Stancato, A., De Luca, C., Pastore, S., and Korkina, L. (2013) Modulation of human keratinocyte responses to solar UV by plant polyphenols as a basis for chemoprevention of non-melanoma skin cancers. *Curr Med Chem* **20**, 869-879

261. Keller, N. P., Turner, G., and Bennett, J. W. (2005) Fungal secondary metabolism - from biochemistry to genomics. *Nat Rev Microbiol* **3**, 937-947

262. Calvo, A. M., Wilson, R. A., Bok, J. W., and Keller, N. P. (2002) Relationship between secondary metabolism and fungal development. *Microbiol. Mol. Biol. Rev.* **66**, 447-559

263. Mishra, B. B., and Tiwari, V. K. (2011) Natural products: An evolving role in future drug discovery. *Eur. J. Med. Chem.* **46**, 4769-4807

264. Lin, T. Y., Huang, C. P., Au, L. C., Chang, Y. W., Hu, C. Y., and Lin, S. B. (2012) A cysteine-reactive alkyl hydroquinone modifies topoisomerase IIalpha, enhances DNA breakage, and induces apoptosis in cancer cells. *Chem. Res. Toxicol.* **25**, 2340-2351

265. Hasinoff, B. B., Wu, X., Krokhin, O. V., Ens, W., Standing, K. G., Nitiss, J. L., Sivaram, T., Giorgianni, A., Yang, S., Jiang, Y., and Yalowich, J. C. (2005) Biochemical and proteomics approaches to characterize topoisomerase IIalpha cysteines and DNA as targets responsible for cisplatin-induced inhibition of topoisomerase IIalpha. *Mol. Pharmacol.* **67**, 937-947

266. Chen, Y. T., Collins, T. R., Guan, Z., Chen, V. B., and Hsieh, T. S. (2012) Probing conformational changes in human DNA topoisomerase IIalpha by pulsed alkylation mass spectrometry. *J. Biol. Chem.* **287**, 25660-25668